PHARMACOKINETICS OF ATARACTIC DRUGS

E. VAN DER KLEIJN

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Promotor : Prof. Dr. J. M. VAN ROSSUM

Pharmacological Institute
Geert Grooteplein Noord 21
University of Nijmegen
the Netherlands

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE WISKUNDE EN NATUURWETENSCHAPPEN AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG VAN DE RECTOR MAGNIFICUS MR. S.F.L. BARON VAN WIJNBERGEN, HOOGLERAAR IN DE FACULTEITEN DER RECHTSGELEERDHEID EN DER SOCIALE WETENSCHAPPEN, VOLGENS BESLUIT VAN DE SENAAT IN HET OPENBAAR TE VERDEDIGEN
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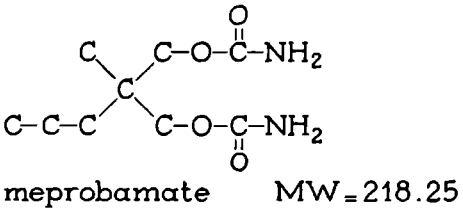
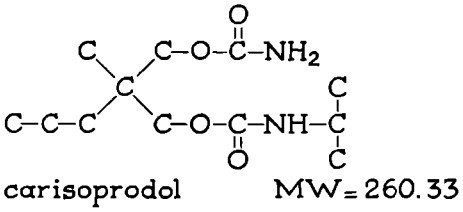
INTRODUCTION

The abundant and still increasing use of psychosedatives in clinical and general practice stimulated the investigation of the pharmacokinetics, chemical analysis and clinical pharmacy of some of the frequently used drugs in the relief of neurotic, anxious, aggressive or mentally strained patients. Because of their central muscle relaxing and anticonvulsive properties these drugs are also used in surgical premedication and in the clinical treatment of epilepsy.

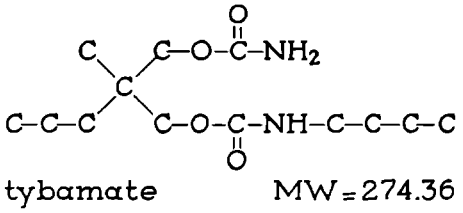
The simultaneous introduction of many modern types of psychotropic drugs in the early fifties promoted the research to drug design, therapeutic application and metabolism of new compounds. Meprobamate was the first compound of a new group of psychosedatives to replenish the classical barbiturates, bromides, bromcarbamides and chloral derivatives. This group is distinguished of the group of the neuroleptics such as chlorpromazine, haloperidol etc., which are primary used in the



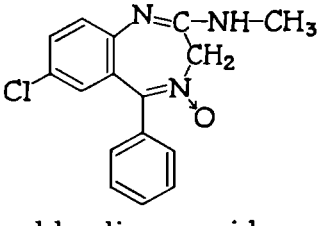
meprobamate MW = 218.25



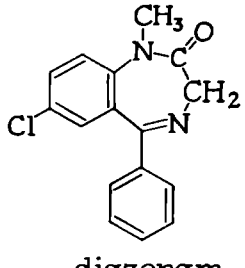
carisoprodol MW = 260.33



tybamate MW = 274.36



chlordiazepoxide
MW = 299.8



diazepam
MW = 284.7

FIG. 1

treatment of the more severe psychoses. The term ataractic is now reserved for the tranquillizers enclosing the subcategories of the meprobamate-and diazepine-group (FIG. 1). In addition some barbiturates and non barbiturates: scopolamine and diphenylmethane-derivatives (hydroxyzine) exhibit ataractic properties. The ataractics have a calming effect at doses that do not yet produce sedation or drawsiness. A further characteristic is their antianxiety effect in animal and man.

The purpose of this investigation was to study kinetics of distribution, elimination and metabolism of the main representatives of the ataractics. On the basis of differences in physicochemical properties of the ataractic compounds variations in kinetics of distribution were anticipated. Kinetics of ataractic drugs in man can only be derived from plasma concentration data, since hardly any drug is excreted in the urine.

The complexity of the relations between drug concentrations in plasma and at possible sites of action promoted the distribution studies in mice. Whole body autoradiography was chosen because of the advantages to detect regional presence of drugs. In addition of pharmacochemical separation techniques the contribution of the metabolic processes could be followed. The uniformity of a population of mice enabled a greater reproducibility than in greater animals.

For the accurate estimation of pharmacokinetic parameters specific and sensitive analysis techniques and many concentration data are necessary. Frequent sampling of blood is practically not feasible in man. For a more accurate determination of the fate of the drugs in the living body the dog was chosen to estimate the concentration-time course with radio-tracer methods.

In general, protein binding is considered as an important factor in elimination and distribution of drugs. Protein-binding capacity was studied both "*in vivo*" and "*in vitro*", in dog and man.

From literature some data were available on the fate of diazepam in man. However, the reported kinetic values differed. Considerably a half-life of about 30 h will give rise to accumulation during chronic treatment. Diazepam and its major metabolite were simultaneously investigated in plasma after a single dose and during chronic treatment on a fixed dosage regimen.

CHAPTER I

PHARMACOKINETICS

INTRODUCTION

The study of "pharmacokinetics" includes the investigation of processes involved in concentration-time relationships of drugs in human and animal bodies (DOST, 1968; KRÜGER-THIEMER, 1968).

When a drug is introduced into the body, many rate-dependent processes can take place before drug molecules and receptors interact and the principle aim of the administration : the therapeutic effect, is reached. Only in a few cases the compartment into which the drug is administered is equal to the biophase or, in other words, the compartment of action, such as for instance for plasma expanders.

The biological object can be considered as a multicompartment system. A compartment is defined here as a mathematical entity, which can have biological similarities. The distribution of a drug over a complex multicompartment system is determined by various transport functions for directly related compartments.

The term compartment is not restricted here to the anatomically and physiologically well defined and imaginable body fluid and tissue compartments, such as intra- and extracellular fluid. Also proteins and other constituents of the biological object, able to bind drug molecules, can be referred to compartments since their interaction with drug can be interpreted by mathematical expressions.

FATE OF DRUGS IN THE TOTAL BODY

After administration of a dose $Q_A{}^0$ of a drug into the body, the fate of this drug can be represented by the scheme in Fig. 1. This scheme is a very simplified expression of the complex sequence of processes taking place in the living body.

What is indicated by one compartment, one line, one rate parameter might at closer view be dissected into many compartments, lines, etc.

The reaction of drug molecules with receptors is mainly considered as an equilibrium reaction and can be expressed mathematically by the mass-action law and the Langmuir adsorption isotherm (ARIENS *et al.*, 1964).

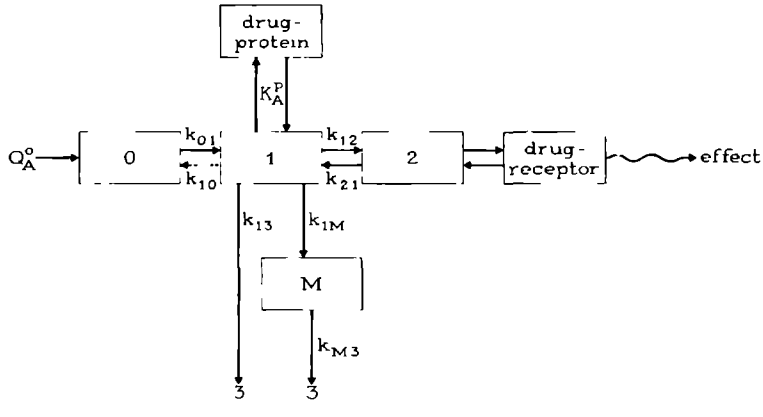


FIG. 1

Schematic representation of the kinetic processes considered in this study. The dose Q_A^0 is introduced into the absorption site compartment (0) from which rate depending processes influence the exchange with the central compartment (1) where k_{01} — absorption rate constant. k_{10} can be considered when enteral circulation takes place Elimination of drugs from the central compartment is possible by *a*) excretion to the infinite volume 3, *b*) by exchange with compartment (2), *c*) by metabolism (compartment M) and *d*) by protein binding. Although much more refinements can be made and more compartments can be introduced which complicate the mathematical treatment, drug concentration-time data can often be interpreted in one or two compartment kinetics. The protein binding capacity can be represented by an association constant according to the mass-action law. Metabolism can be treated as a separate compartment since the rate of metabolite appearance in the compartment of analysis can also be interpreted as a first order process. Drug receptor interaction is not studied.

Many factors in the living organism mediate this drug-receptor interaction; there is a "dynamic equilibrium". These factors play a role in the appearance and disappearance of a substance or in the words of DOST (1968): "in the disturbance and recovery of the dynamic equilibrium". Therefore factors such as diffusion, passive transport, filtration, local distribution, adsorption, chemical reaction, rough mechanical convexion influence, alone or combined, the kinetic processes of distribution of either a physiological or pharmacological compound.

In principle all these processes can separately be considered in mathematical terms. Some processes may dominate the observed phenomena while others can be neglected. It is therefore often allowed to summarize the experimental data of the drugs in the body in simple terms.

Presence of compartments

Most drugs, except the intravascularly administered ones, are mainly absorbed by passive diffusion across membranes which function as lipid barriers.

The characteristics of the membranes, which are considered as semi-permeable, vary from organ to organ and from tissue to tissue, even within subcellular constituents. One may say that the characteristics of the membranes differ from compartment to compartment. The passage of drugs through membranes can be expressed by rate constants. Thus, anatomically distinguished body constituents may have mathematical resemblances.

It is known that substances with low lipid/water partition coefficients usually show poor membrane penetration, whereas lipophilic substances with corresponding high lipid/water partition coefficients usually show fast and efficient passage (Butler, 1950). Exceptions to this rule are the low molecular weight lipid-insoluble substances, such as urea and water.

Body membranes may be viewed as lipoid barriers periodically interrupted by small water-filled channels or pores (Doluisio and Swintosky, 1965).

Certain barriers, such as those existing between blood and brain, will allow the passage of only highly lipid-soluble substances, while other membranes, such as those existing between blood capillaries and the extracellular space are porous and allow the passage of certain macromolecules.

Transport of drugs

To reach the so-called biophase or receptor compartment, the drug molecules are designated on an available transport medium or, in the simplest case, on free diffusion from the dosage compartment through various other compartments (Fig. 1).

The transport can be distinguished in : 1. passive transport, 2. active transport, 3. special mechanisms, 4. transport by body fluids.

1. As far as the distribution over the multicompartment system is based on free diffusion of the drugs, the overall physicochemical properties such as hydrophility, lipophility, ionic character, polarity, size and shape are determinant factors (Ariëns, 1968[1]).

2. Active transport of drugs is often qualified as such by the need of energy, supplied by metabolic, enzymatic processes. One may distinguish

free diffusion and carrier-bound diffusion. In the case of free diffusion Fick's law can be applied and here, in principle, a straight proportionality between diffusion velocity and concentration gradient is observed. There is no preference in direction. In the carrier-bound diffusion one step in the biological sequence of the system can be a rate-limiting factor. Saturation of the carrier system and a maximum in the transport velocity can occur. The system is, in general, specific for certain compounds. Phenomena of competition for the carrier system are observed. Mainly a preferred direction in the membranes and transport against a concentration gradient can be distinguished (ARIËNS, 1968[2]).

3. Uptake of substances in the cell by phagocytosis and pinocytosis and the release by reversed pinocytosis are phenomena reported to histological observations. In this case no membranes are directly involved (LASTER and INGELFINGER, 1961).

4. Drugs can be transported from the site of absorption through the body by blood, lymph or cerebrospinal fluid. Compounds in blood can be transported either dissolved in the water fraction and when bound, by the protein fraction. Water-insoluble, lipophilic compounds are mainly protein-bound (see Chapter 2). There is an apparent correlation between lipid/water partition coefficients and protein binding within a series of compounds (GOLDBAUM and SMITH, 1954; DOUGLAS *et al*, 1964; VAN DER KLEIJN, 1969).

Intravascularly administered, at the physiological pH insoluble drugs are frequently introduced into the body as highly concentrated and solubilized liquids or even in crystalline form. At least, when introduced into the body in a small volume many drugs might crystallize in the area of administration at the pH of the body fluid. Proteins will restrict crystal growth. While the concentration decreases plasma proteins behave as stabilizers or solubilizers for the saturated solution.

The term protein binding should be reserved to those phenomena in which the interaction between drug molecules and proteins can be expressed according to the mass-action law, in other words, when one can distinguish at least mathematically one or more classes of binding sites on the receptive molecule. The degree of protein binding is concentration dependent in the sense that the protein-binding capacity is high at low drug concentration. In the case of solubilizing, phenomena other than those involved in protein binding will play a role (Chapter 2). When no concentration dependence can be observed, physicochemical partition between aqueous and micellar phase must be considered.

Relations between drug concentrations in body compartments and biological activity

Since the very beginning of drug therapy the relationship between the dose and its activity has been well-understood. The study of concentration-activity relationships, which is called "pharmacodynamics", has only with few exceptions been integrated into pharmacokinetics. Relationships between concentrations and activity in living individuals is often intricate. In general after a single dose the maximum blood level of the drug does not coincide with the maximum tissue levels, nor the time of maximum biological activity. Theoretical predicting of concentration-time relationships of drugs in various body compartments by a kinetic approach does not necessarily delineate the pharmacological activity-time relationships (Levy, 1964, 1966, 1967[2]; van Rossum and van Koppen, 1968; Segre, 1967; Nelson, 1961; Maickel *et al.*, 1969).

Nevertheless, the therapeutic activity of a drug is considered to be controlled by its concentration at the site of action, in the biophase or the receptor compartment. This concentration is assumed to be closely related to the concentrations in the other compartments, one of them being the blood.

Many fundamental studies have been made on the relation between concentration and activity in isolated systems (Ariëns, Simonis and van Rossum, 1964).

By plotting the intensity of a well defined effect against the applied dose similar curves of several drugs are obtained. In general the effect will asymptotically reach a maximum value. Increasing the dose when the maximum effect is reached will not result in a greater effect. Over a restricted concentration range the increase in the effect is directly proportional to the logarithm of the dose. However, in an individual it has often been impossible to choose the right criteria and circumstances to demonstrate such a reproducible concentration-activity relationship.

Kinetics of elimination

In this study mainly first-order kinetics are considered. In many cases the concentration-time course of drugs in plasma appears to decrease rapidly at first and thereafter more slowly as the amount in the body declines. The rate of disappearance is directly proportional to the concentration at each moment.

This means that

$$dQ_A/dt = -kC_A \tag{1}$$

where : Q_A = amount of drug A present in the body (mg or mole), k = clearance rate constant (l/h, ml/min) and C_A = concentration of the drug (mg/l, μg/ml, M).

C_A is equal to the amount of drug (Q_A) divided by the volume (V) of the compartment over which it is uniformly distributed.

Integration of equation (1) leads to the exponential expression

$$C_A{}^t = C_A{}^0 e^{-t/\tau} \tag{2}$$

where : $C_A{}^t$ = concentration of drug A in plasma at time t, $C_A{}^0$ = concentration of drug A initially present, and τ = the time constant for elimination.

$$\tau = V/k \tag{3}$$

A theoretical concentration-time curve is graphically represented in Fig. 2A. According to equation (2), a straight line is obtained when the logarithms of the drug concentration are plotted against the time (FIG. 2B).

The time constant is the important parameter of the disappearance

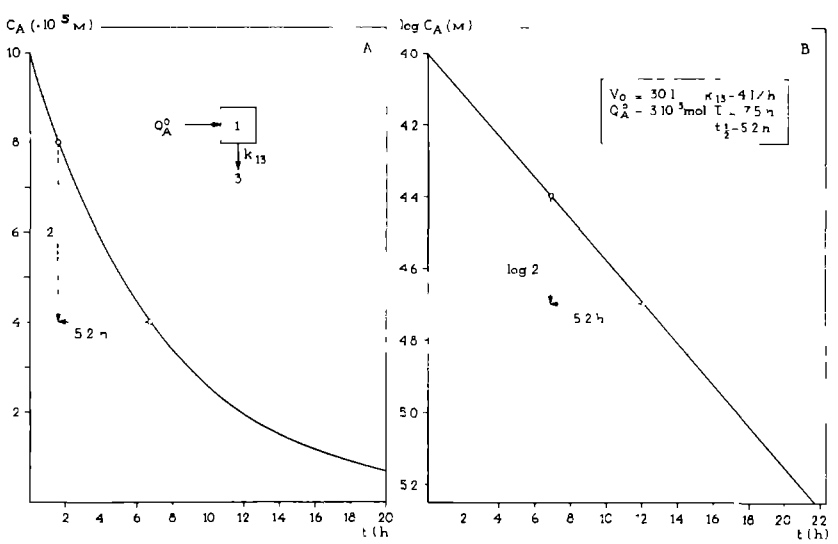


FIG. 2

A. Exponential decline of the plasmaconcentration represented in a linear diagram according to the scheme of a one-compartment open system.

B. The logarithm of the plasmaconcentration declines according to a straight line.

process. τ is directly related with the biological half-life time ($t_{\frac{1}{2}}$) according to :

$$t_{\frac{1}{2}} = 0.693 \times \tau \qquad \text{or} \qquad \tau = 1.44 \times t_{\frac{1}{2}}. \tag{4}$$

The drug concentration can analytically be determined at any desired time, so that $t_{\frac{1}{2}}$ and τ can easily be calculated from the log concentration-time curve.

C_A^0 can be found with sufficient accuracy as the intercept of the curve with the ordinate of the log concentration-time line.

This presentation will only fit experimental data when the achievement of the equilibrium situation is rapid compared to the disappearance or elimination process. This is often the case after intravenous administration or when the absorption is very rapid compared to the elimination process.

$t_{\frac{1}{2}}$ and τ do not give information about the mechanism of drug disappearance. Since $\tau = V/k$ the disappearance can be controlled either by the clearance rate constant, by the distribution volume or by both. The distribution volume and the clearance rate constant are therefore very important parameters in pharmacokinetics.

The fictive distribution volume

The fictive distribution volume is for more reasons an important factor in pharmacokinetics since it has been shown that its value is nearly constant irrespective of the applied dose as soon as the dynamic steady state is reached (Dost, 1968).

In the simple case of exponential elimination of the drug V can be found from :

$$V = Q_A/C_A^0 \tag{5}$$

The distribution volume may have physiological resemblance. When the drug is uniformly distributed over for instance the total body water or the extracellular space, plasma concentrations might reflect a physiologically distinguished body fluid compartment.

Distribution studies of drugs with the whole body autoradiographic technique, often demonstrate an unequal distribution (Ullberg, 1958). In general, however, the value for V has no physiological or anatomical resemblance.

Since several tissues show different affinities for the drug and specific variations in exchange rate will govern the distribution process, it is

better to introduce the term fictive or apparent distribution volume (V_f). V_f can be considered according to the following relation :

$$V_f = V_1[1 + (k_{12}/V_1)/(k_{21}/V_2) + \cdots + (k_{1N}/V_1)/(k_{N1}/V_N)] \qquad (6)$$

where V_f = fictive distribution volume, V_1 = plasma volume, V_2 = fictive volume of a peripheral compartment and V_N = fictive volume of compartment N.

When the value of V_f is known, information becomes available about the mechanism of drug elimination and clearance (RIEGELMAN *et al.*, 1968[2]).

The distribution volume is predominantly controlled by the physicochemical properties of the compound and the receiving tissue (RIEGELMAN *et al.*. 1968[1]). For one drug individual variations can be expected.

Generally the pharmacological influence of the drug on the physiological conditions of distribution and elimination is neglected. In some cases the action of the drug (e.g. diuretics) changes the distribution or elimination processes. It appears that the amount of drug in the various tissues and compartments can differ greatly from that in the blood, while in other tissues the amount may be equal at the same time.

The distribution volume is an important factor to estimate optimal dosage regimens of patients. A common method derives the parameters necessary for calculation from plasma concentration data after administration of a single dose (BÜNGER *et al.*, 1961; WAGNER, 1969). However, it is possible that no steady state will be achieved after a single dose (DE SILVA *et al.*, 1966). It is more reasonable to estimate drug levels after repeated dosage and to determine the rate of accumulation (VAN ROSSUM and TOMEY, 1968; Chapter 7). Regular estimations of plasma concentrations enable the detection of over-, under- or correct dosage regimens. If necessary correction can then be made during chronic treatment.

When the drug in the central compartment rapidly and extensively exchanges with a small, but for drug action or distribution highly specific peripheral compartment, oscillations of drug concentrations in this compartment will seldom be recognized in the central compartment. Since k_{1N}/k_{N1} is high, variations in drug concentrations will have great influences in the small peripheral compartment. The clearance ratio is often unknown, so blood concentrations will give no information about the concentration in the specific peripheral compartment.

Analysis of concentrations in tissues and organ homogenates where the site of action is supposed to be located is also often insufficient to detect the presence of the drug in a small specific compartment or biophase.

The difficulties involved in the correlation of the amount of drug in the plasma with the amounts in other compartments lead to the selection of macroautoradiography in intact animals as a tool to distinguish the drug at regions which are impossible to follow in living animals or men.

In this section disappearance of the drug is considered according to a monophasic exponential elimination which assumes the instantaneous and uniform presence of the drug. At least absorption is so rapid that no influence on the elimination process can be demonstrated.

Many factors, however, can influence the establishment of the dynamic equilibrium situation. The rapidly injected fluid will be mixed over the total plasma volume which will take about 5-15 minutes before a steady state is reached. At the same time exchanges with the extracellular, intracellular and other possible compartments may occur. The rate of this exchange is determined predominantly by the physicochemical properties of the drug. There are also physiological factors influencing the slope of this first part of the concentration-time curve. These factors can be mediated by the drug, by the reaction of the body to the injection, the viscosity of the administered substance, the state of vessels and capillaries, the vascularity of the tissues, etc. When the initial equilibration period shows its influence on the second phase of the curve a two- or multicompartment system can be considered (ROWLAND and RIEGELMAN, 1968; this chapter, page 17). Multi-exponential equations are necessary to interpret the concentration-time course.

KINETICS OF ABSORPTION

In pages 13 and 14 factors are discussed concerning the direct administration of drugs into the blood circulation. All other routes of administration will take time before the transport medium is reached.

The extent and the rate of absorption are of great importance to drug action. They determine the magnitudes of plasma and tissue concentration with respect to time and hence, the onset, intensity and time course of biological activity. Many factors influence the rate and the extent of the passage of drugs from the absorption site compartment to the body fluids. The several routes to administer drugs will not be discussed.

Absorption of drugs may be considered as a first-order process. Under the assumption that the drug is not eliminated the following equation is derived, representing the concentration in the body ($C_A{}^t$) as a function of time (t):

$$C_A{}^t = (Q_A{}^0/V_f)(1 - e^{-t/\tau_1}) \qquad (7)$$

where: τ_1 = time constant of absorption.

In this model the plasma concentration increases to a maximum level when complete absorption is reached.

When simultaneous exponential elimination of the drug takes place in addition to the first-order absorption process and no elimination by excretion occurs, the elimination term can be included.

$$C_A^t = (Q_A^0/V_f)[\tau_2/(\tau_2 - \tau_1)](e^{-t/\tau_2} - e^{-t/\tau_1}) \qquad (8)$$

where : τ_2 = time constant of elimination.

From Fig. 3 it may be clear that the intercept of the extension of the elimination curve with the ordinate, M,

$$M = (Q_A^0/V_f) \cdot [\tau_2/(\tau_2 - \tau_1)]$$

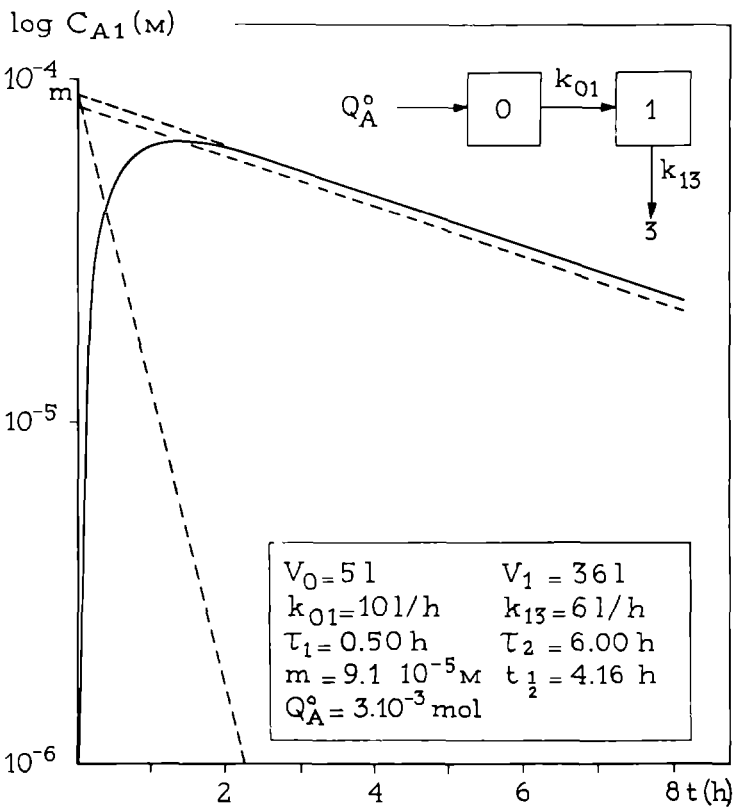


FIG. 3

Curve representing the logarithm of the plasmaconcentration after oral administration when absorption and elimination simultaneously take place according to first-order processes. The intercept of the extension of the elimination curve with the ordinate gives M

cannot simply be treated as the fictive initial drug concentration C_A^0, thus allowing the calculation of the distribution volume (equation 6) since :

$$C_A^0 = M(\tau_2 - \tau_1)/\tau_2 \tag{9}$$

When τ_1 is small compared to τ_2, M is equal to C_A^0 and the data can be interpreted in a one compartment system. The course of the concentration is represented in Fig. 3.

When the drug is transported to the absorption site compartment, which is probable when enteral circulation occurs, the plasma concentration follows a second-order process.

Kinetics of dissolution

In many cases drugs are administered as active substances mixed with inactive additives. Wherever the place of application will be, the rate of release of the active compounds from the additives or, when no additives are used, from the dosage must be considered. Since the release can be expressed in mathematical terms we will consider the dissolution of the drug and its influence on the absorption site compartment as a specific compartment : the drug dosage compartment.

The equation originally introduced by Noyes and Whitney in 1897 for benzoic acid generally covers the phenomena affecting the dissolution of drugs :

$$C_A^t = C_A^s(1 - e^{-t/\tau_D}) \tag{10}$$

where : τ_D = time constant of dissolution (h, min), C_A^s = concentration of saturated solution of the drug (mg/ml, g/l, M), and C_A^t = concentration of the solution at time t (mg/ml, g/l, M).

The experimental work of Nelson (1957) has led to the following equation for the dissolution of drugs :

$$Q_A^t = KSC_s t \tag{11}$$

where : Q_A^t = the amount dissolved, K = the rate constant with dimensions distance/time, S = the surface area and C_s = the concentration of dissolving solid at the solid side of the diffusion layer.

K is controlled by physical state of formulation, particle size, crystal shape and solubilization tendency of the particle surface influenced by secondary forces, intensity of agitation, temperature, etc. The absorption equation (7) can be read as :

$$C_A^t = KSC_s t(1 - e^{-t/\tau_1})/V_f \tag{12}$$

However, factors other than dissolution rate are involved in the availability of drug molecules for absorption. They can be either physical or physiological.

Chemical stability in the physiological environment, chemical modification by complex formation or conversion influenced by the environment, pH and hence, the degree of ionization, and the partition coefficient are important physical factors in the absorption characteristics of the drugs.

Physiologically, surface and motility (peristaltic activity) of the absorption area, vascularity and bloodflow rate, stomach emptying, which may be influenced by the drug itself or by the condition or the position of the patient, enteral circulation, and metabolizing micro-organisms at the site of absorption can have a retarding or accelerating influence on drug absorption (WAGNER, 1961; LEVY, 1967 (1); RIEGELMAN *et al.*, 1968 (1); SCHELINE, 1968). Rate of release from the dosage is important when sustained absorption is intended (WIEGAND and TAYLOR, 1960; KRÜGER-THIEMER and ERIKSEN, 1966).

KINETICS OF METABOLISM

When biotransformation is an important factor in the elimination of a drug and when the appearance and elimination of the metabolite(s) can also be treated as first order processes, the system can be interpreted according to Fig. 4A*a*. The following differential equation for the metabolite(s) can be formulated :

$$dQ_M/dt = k_{1M}C_{A1} - k_{M3}C_M \tag{13}$$

After integration the following equation is derived :

$$C_M{}^t = (Q_A{}^0/V_M)[\tau_{2M}/(\tau_{2M} - \tau)][k_{M3}/(k_{M3} + k_{13})](e^{-t/\tau_{2M}} - e^{-t/\tau}) \tag{14}$$

where : k_{m3} = clearance rate constant for metabolite (l/h), k_{13} = clearance rate constant for parent drug (l/h), τ_{2M} = time constant of metabolite elimination. The time constant of elimination of parent drug (τ) = time constant of metabolite appearance (VAN ROSSUM, 1968[1]).

Since the fictive distribution volume is dependent of, among other factors, the physicochemical properties of the compound this value may differ for drug and metabolites.

k_{13} will also be controlled by other physicochemical properties such as pk_A-value, lipophilic nature and protein binding. Fig. 4A and B give two theoretical examples for cases when the clearance constants of the

drug and its metabolites are varied. Fig. 4C shows the theoretical case of the consequences of repeated administration, when the metabolite manifests a much slower elimination than the parent compound. Accumulation of the metabolites takes place.

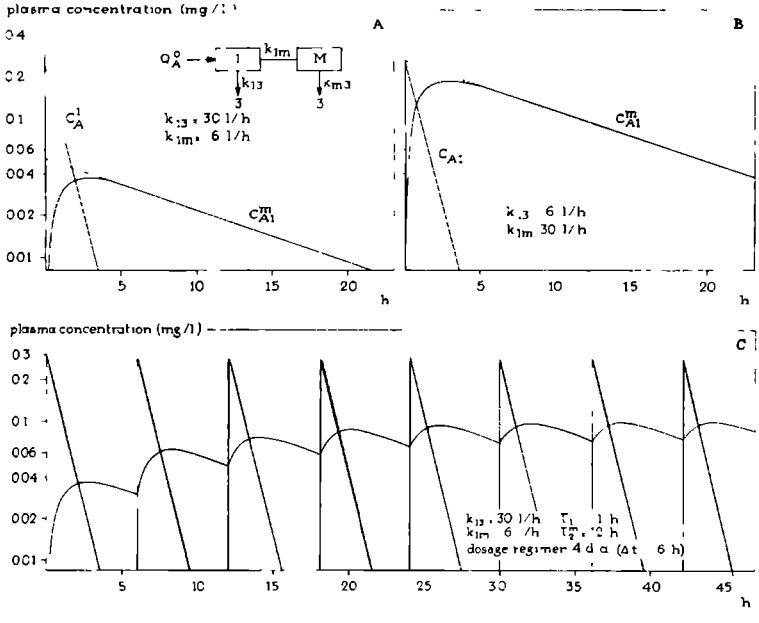


FIG. 4

4Aa — (insertion) Schematic representation of the kinetic processes involved in the elimination of a dose Q_A^0 by metabolism and excretion of drug and metabolite.

4A — Log plasmaconcentration-time curves of drug A (Q_A^0 — 10 mg) and its metabolite M when elimination is controlled by excretion (k_{13} = 30 l/h) and by biotransformation at a slower rate (k_{1M} = 6 l/h). Excretion of metabolites is relatively slow (k_{M3} = 3 l/h). The fictive distribution volumes of, both, drug and metabolite are equal ($V_1 = V_M$ = 36 l); (τ_1 = 1, $t_{\frac{1}{2}}$ = 0,69, τ_{2M} — 12 h).

4B — Log plasmaconcentration curves of drug A and metabolite M under the same conditions as in Figure 4A. Here the excretion clearance of the drug is slow (k_{13} — 6 l/h) and the metabolic clearance is high (k_{1M} = 30 l/h). The excretion of the metabolite is again slow (k_{M3} = 3 l/h).

4C — Log plasmaconcentration pattern of drug A and metabolite M under the same conditions as in Figure 4A after repeated dosage (4 d.d. 10 mg). As a consequence of the great time constant τ_{2M} of the metabolite, accumulation will occur while accumulation of the parent drug can be excluded. The maximum level of the metabolite reaches its maximum after about 30 h. The maximum plateau level is 2 ½ times higher than the maximum level after a single dose.

The curve corresponding to the sum of the molar concentrations of the parent drug and the metabolite will be represented by :

$$C_s^t = C_A^t + C_M^t \qquad (15)$$

Depending on the chosen parameters this curve has much in common with the curve of total radioactivity found in the experiments on the meprobamate-group (Chapter 6).

Protein binding

There is sufficient evidence that it is not the concentration of a drug in the plasma, but the concentration in the plasma water : the unbound, so-called free drug concentration that is of direct importance for the exchange with the tissues and for elimination (Krüger-Thiemer *et al.*, 1964; Krüger-Thiemer, 1968). Many drugs, organic as well as inorganic, can bind to proteins. From the therapeutic point of view plasma proteins can function as a depot.

C_{Af}, the free plasma concentration of a drug controls the concentration gradient, when transport to other compartments is achieved by free diffusion.

The fictive distribution volume (V_f) must be calculated on the basis of C_{Af} :

$$V_f = Q_A^0/C_{A_f} \tag{16}$$

Protein-binding capacity is concentration dependent for many drugs. The capacity will decrease at higher concentrations when primary classes of binding sites are saturated.

When drug-protein interaction can be described according to the mass-action law the relation between free drug concentration and the total plasma concentration (C_A) reads :

$$C_{A_f} = [(K_A{}^P C_{A_f} + 1)/(K_A{}^P C_{A_f} + nK_A{}^P P_t + 1)]\, C_A \tag{17}$$

where : $K_A{}^P$ = association constant, P_t = total concentration of protein with binding capacity and n = number of independent binding places.

In the field of ataractic drugs of the meprobamate- and diazepine-group no concentration dependence could be demonstrated in physiological circumstances over the therapeutically possible concentration range. Here the fictive distribution volume on the basis of C_{Af} will be found by multiplying C_A with a simple factor (Chapter 2).

$$V_f = (100/\%\ \text{unbound})(Q_A^0/C_A) \tag{18}$$

Accumulation

When drugs are chronically administered to patients, which is very often the case in, for instance, chemotherapy, cardiology, endocrinology

and in psychiatric practice, accumulation has to be considered. Theoretically accumulation will occur when drug administration is repeated before the previous dose is eliminated (FIG. 5).

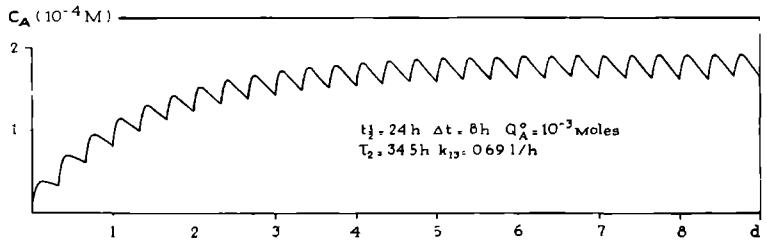


FIG. 5

Log concentration-time curve of a drug after repeated dosage at intervals (Δt) much shorter than the biological half-life ($t_{\frac{1}{2}}$) of elimination. Accumulation till a maximum level takes place.

In many cases a continuous effect of the drug is desired and dosage schedules are designed by trial and error to enable the adjustment of a constant therapeutically effective drug concentration level and response. It will be evident that this level will oscillate around a mean level depending on dose, route of administration, dosage interval, biological half-life time of elimination, dosage form, simultaneous drug supply, etc.

A single dosage may often be insufficient for the achievement of the steady state and thus for reaching the constant individual value for V_f. The rate of accumulation depends merely on the half-life of elimination. The mean plateau plasma concentration of a drug A ($\bar{C}_{Apl}$) can be represented by a simple formula assuming the operation of first-order kinetics and complete absorption :

$$\bar{C}_{Apl} = 1.44(Q_A^0/V_{fA})(t_{\frac{1}{2}A}/\Delta t) \tag{19}$$

and for metabolites (M) :

$$\bar{C}_{Mpl} = 1.44(Q_A^0/V_{fM})(t_{\frac{1}{2}M}/\Delta t) \tag{20}$$

(WAGNER, *et al.*, 1965; VAN ROSSUM and TOMEY, 1968).

When dosage is repeated at fixed periods equal to the half-life time of elimination ($\triangle t = t_{\frac{1}{2}}$), it has been calculated that :

$$t_{ac\frac{1}{2}} = t_{\frac{1}{2}}[1 + 3.3.\log \tau_2/(\tau_2 - \tau_1)] \tag{21}$$

where : $t_{ac\frac{1}{2}}$ = time of reaching 50 % of the plateau level, τ_1 = time constant of absorption, τ_2 = time constant of elimination (VAN ROSSUM, 1968[2]). Drugs with a long half-life of elimination show the strongest

accumulation capacity. In those cases $\tau_2 \gg \tau_1$ and, hence $t_{ac\frac{1}{2}} = t\frac{1}{2}$. This means that after a period of 3 times $t_{ac\frac{1}{2}}$ 87.5 %, after 4 times more than 93 % and after 5 times more than 96 % of the mean accumulation plateau level is reached. Fig. 4C shows an example of the case for a metabolite with a different $t\frac{1}{2}$ than the parent drug. Thus while the parent drug does not accumulate, the metabolite reaches a plateau level.

MULTICOMPARTMENT SYSTEM

In many cases it appears that a one-compartment system is a too simple approximation of the phenomena which take place after introduction of a drug into the body. When many analytical data are collected the logarithmic plasma concentration curve may show a bi- or multiphasic pattern. This curve can be interpreted by assuming the distribution of the drug over more than one distribution volume (ROWLAND and RIEGELMAN, 1968).

Biphasic, exponentially declining plasma curves can be interpreted in terms of a two-compartment open system as represented in Fig. 6A*a*.

When a drug is introduced without delay into the central compartment (1), then at time o the amount of drug in the body is equal to the dose $Q_A{}^0$. Exchange with the second or peripheral compartment takes place. The exchange processes may be presented by a series of linear differential equations :

$$dQ_{A1}/dt = -k_{12}C_{A1} - k_{21}C_{A2} - k_{13}C_{A1} \tag{22}$$

$$dQ_{A2}/dt = k_{12}C_{A1} - k_{21}C_{A2} \tag{23}$$

$$dQ_{A3}/dt = k_{13}C_{A3} \tag{24}$$

where : k_{12}, k_{21}, k_{13} are the clearance rate constants (ml/min; l/h) and Q_{A1}, Q_{A2}, Q_{A3}, are the amounts of drug in the central and peripheral compartment and the amount excreted.

After integration the following equations are obtained for the concentration of the drug in the central compartment (C_{A1}), the quantity in the peripheral compartment Q_{A2} and the quantity eliminated Q_{A3} :

$$C_{A1} = L_1e^{-t/\tau_1} + L_2e^{-t/\tau_2} \tag{25}$$

$$Q_{A2} = MV_2(e^{-t/\tau_2} - e^{-t/\tau_1}) \tag{26}$$

$$Q_{A3} = Q_A{}^0 - N_1e^{-t/\tau_1} - N_2e^{-t/\tau_2} \tag{27}$$

in these relations :

$$1/\tau_1\tau_2 = (k_{13}/V_1)(k_{21}/V_2) \tag{28}$$

$$1/\tau_1 + 1/\tau_2 = k_{12}/V_1 + k_{13}/V_1 + k_{21}/V_2 \tag{29}$$

From the experimental curves it is possible to estimate directly τ_2 and L_2 (FIG. 6A). The residues of the extension of the second phase from the

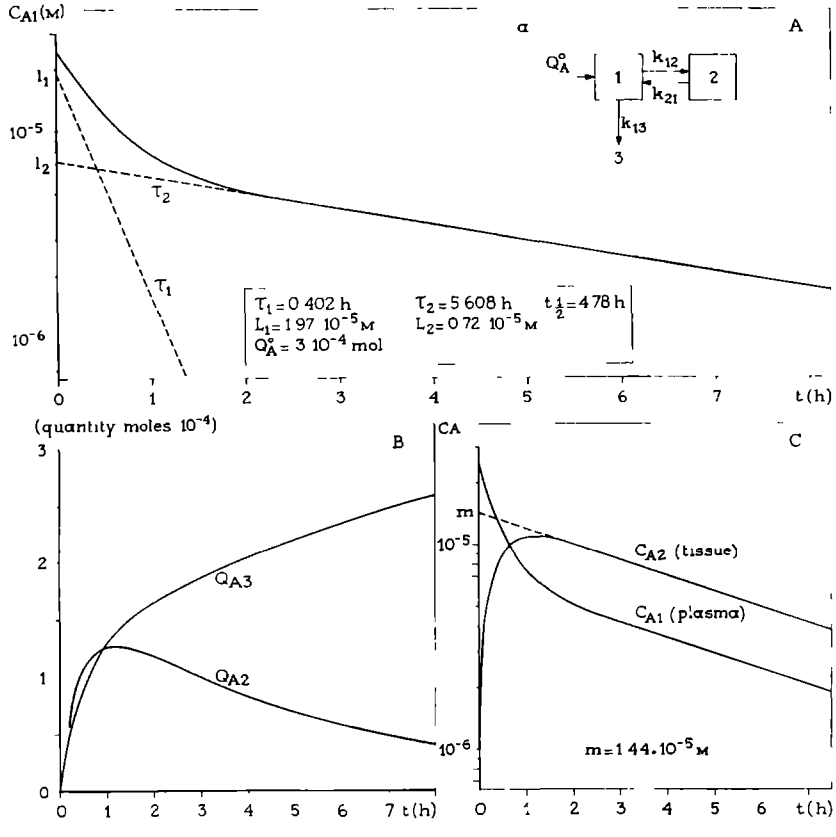


FIG. 6

6Aa — Schematic representation of a two compartment open system.

6A — Log concentration-time curve of a drug after a single dose (Q_A^0). From the course of the second phase of the curve, τ_2 and L_2 can be found. The residue of the extension of this phase from the total curve gives another straight line from which τ_1 and L_1 can be found.

6B — The data in A allow the calculation of the course of the amount of drug in the peripheral compartment (Q_{A2}) and of the excreted amount (Q_{A3}).

6C — When the clearance ratio is known it is possible to calculate the fictive distribution volume of the peripheral compartment (V_2) and the course of the concentration C_{A2}. ($V_1 = 12$ l; $V_2 = 24$ l; $k_{12} = k_{21} = 16$ l/h; $k_{13} = 12$ l/h).

total biphasic curve give a new line from which τ_1 and L_1 can be calculated. These data allow the calculation of V_1, k_{12}, k_{13}, k_{21}/V_2 and hence, MV_2 and N_1 and N_2 according to the following equations :

$$V_1 \quad - Q_A^0/(L_1 + L_2) \tag{30}$$

$$k_{12} = Q_A^0 L_1 L_2(\tau_2 - \tau_1)^2/[\tau_1\tau_2(L_1 + L_2)^2\,(\tau_1 L_1 + \tau_2 L_2)] \tag{31}$$

$$k_{13} = Q_A^0/(\tau_1 L_1 + \tau_2 L_2) \tag{32}$$

$$k_{21}/V_2 = (\tau_1 L_1 + \tau_2 L_2)/\tau_1\tau_2(L_1 + L_2) \tag{33}$$

$$MV_2 \quad Q_A^0(k_{12}/k_{13})\; V_2/k_{21}(\tau_2 - \tau_1) \tag{34}$$

$$N_1 = Q_A^0[(V_2/k_{21}) - \tau_1]/(\tau_2 - \tau_1) \tag{35}$$

$$N_2 = Q_A^0[\tau_2 - (V_2/k_{21})]/(\tau_2 - \tau_1) \tag{36}$$

$$V_f = V_1[1 + (k_{12}/V_1)/(k_{21}/V_2)] \tag{37}$$

From the dose Q_A^0 and the obtained parameters τ_1, τ_2, L_1 and L_2 it is possible to calculate k_{12}/V_1, k_{12}/V_3 and k_{21}/V_2. Also the amounts present in the peripheral compartment and the amount of the excreted drug can be calculated at any time (FIG. 6B).

Some times it is allowed to consider $k_{12} = k_{21}$, which simplifies equation 34 to equation 38.

$$M = Q_A^0/k_{13}(\tau_2 - \tau_1) \tag{38}$$

and

$$C_{A2} = M(e^{-t/\tau_2} - e^{-t/\tau_1}) \tag{39}$$

From equation 33 it is possible now to calculate a value for V_2. Also the concentration-time course in the peripheral compartment can be calculated (FIG. 6C) with equation 39.

However, for drugs that rapidly penetrate into the intracellular compartment which is the case for the ataractics in our study, the clearance ratio k_{12}/k_{21} may be much greater than 1 and behaves as an apparent distribution coefficient. In this case it is not possible to calculate the values for V_2 and to get information about the concentration course in this peripheral, tissue compartment.

When more phases in the experimental concentration pattern of drugs can be observed more compartments must be taken into account. Highly complicated mathematical terms will be necessary to derive the parameters.

CONCLUSIONS

The quantitative tracing of the original drug in body fluids is important to evaluate reasonable dosage schedules of drugs.

Pharmacokinetic factors like biological half-life time of elimination and distribution volume are necessary to bring more objective criteria into

pharmacotherapy, especially when subjective criteria are difficult to establish.

The biological half-life time is controlled by the magnitude of the distribution volume and the clearance constant k. This parameter can be calculated from plasma concentration curves. The clearance constant can be composed of several constants such as metabolic, renal, alveolar and distribution clearance constants. Depending on the nature of the compounds one of these constants will dominate in general.

The fictive distribution volume of a drug at the equilibrium situation is considered to be constant for a special drug in an individual.

To obtain maximum therapeutic profit and less toxic side-effects for chronically administered, accumulating drugs it is advisable to follow plasma concentrations at regular time intervals.

Analytical problems have frequently prevented the accurate determination of drugs in the presence of biogenous compounds or structurally related metabolites. The introduction of radioisotope techniques in conjunction with chromatographical and electrophoretical separation techniques enabled the qualitative and quantitative tracing of drugs in the body. In the past, pharmacokinetic data of drugs have often been published on the basis of the analysis of drug and metabolites together. The consequence can be confusing when the metabolites show no or modified activity. The disadvantage of many bioanalytical procedures is the disturbance of the physiological conditions. Homogenate-extraction techniques may level out specific locations.

Our investigations deal with the distribution, elimination and biotransformation of ataractic drugs of the diazepine- and meprobamate-group.

Macroautoradiography appeared to be a valuable tool in the tracing of drug and metabolites in analytically difficult attainable regions. The experiments have been carried out in mice. Frequently, data obtained from laboratory animals are difficult to translate to man.

Experiments in dogs have been carried out to get information about the concentration-time course with the accurate radioisotope-dilution analysis. They are intended as an introduction to the tracing of the drugs in man.

In man, plasma is the "closest" body fluid for analysis that can reflect the kinetic processes in the compartment of action. Gaschromatography with sensitive detection enabled the quantitative estimation of the drug over the therapeutic concentration range.

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CHAPTER II

PROTEIN BINDING AND LIPOPHILIC NATURE OF ATARACTICS OF THE MEPROBAMATE- AND DIAZEPINE-GROUP

INTRODUCTION

Physicochemical properties of drugs, such as lipophilic nature, *p*K-values and protein binding are important factors involved in the kinetics of absorption, distribution, and elimination, and therefore in drug action.

Many investigations have been published on the correlations between lipophilic properties of a drug, expressed as partition coefficient between two immiscible liquid phases, and its pharmacological activity (BUECHI and PERLIA, 1960; BRODIE, 1964; FELTKAMP, 1965; HANKE and KLINGER, 1957; HESS *et al.*, 1968).

In the field of barbituric acid derivatives onset and time course of action were successfully correlated with physicochemical properties of the compounds (BUTLER, 1950). As a general rule, "short-acting" barbiturates have a high partition coefficient while the "long-acting" derivatives show a lower partition coefficient. Other factors as acid-base equilibrium in the body (pK_a), dose, route of administration and rate of biotransformation are important.

Many drugs, organic as well as inorganic, are bound to some extent to proteins (GOLDSTEIN, 1949; MEYER and GUTTMAN, 1968). In general, drug-protein interaction is treated as a dynamic equilibrium between the drug molecules which occupy the binding sites and the drug molecules which are free in solution.

Protein binding is generally considered as an important factor in the distribution and elimination of drugs. The free, unbound, drug molecules are presumed to be available for transport to the tissues and hence protein binding controls the diffusion rate and the apparent distribution volume (KRÜGER-THIEMER *et al.*, 1964). Mainly acidic drugs have been considered in these studies (MEYER and GUTTMANN, 1968).

The purpose of this investigation was to study protein binding of

ataractics as a factor in drug distribution, and the character of the binding in relation to the lipophilic nature of these compounds.

Materials and Methods

The following drugs, either cold or ^{14}C-labeled compounds, were investigated with respect to binding to proteins and surfactants, and to partition between organic solvents and water meprobamate, carisoprodol (N-iso-propyl meprobamate, Apesan®), tybamate (N-*n*-butyl meprobamate, Solacen®), chlordiazepoxide (Librium®) and diazepam (Valium®) In drugs of the meprobamate-group the label was situated in the (unsubstituted) carbamate-group, in the diazepine-group the C_2-position was labeled

Binding was studied to dog plasma (heparinized, 5000 IU l "*in vitro*" and "*in vivo*"), to bovine serum albumin (cristallyzed B S A Povite, Amsterdam), to polysorbate 80 (Tween® 80) and to polysorbate 20 (Tween® 20, Atlas Chemical Industries)

Analytical procedures.

1 *Assay of ^{14}C-radioactivity*

1 1 Liquid scintillation counting (LSC)

Radioactivity of protein-containing liquids was counted after mixing with 1 0 ml of the hydroxide of Hyamine 10 × (Packard) or 1 0 ml of 0 50 M Soluene (Packard)

Digestion with Soluene was done by warming for 45 min at 50°C After mixing with 15 0 ml of scintillation cocktail of Bray (1960), counting was performed with a Nuclear Chicago, Mark I, liquid scintillation counter Corrections were made by the external standard method Protein-free and surfactant-containing solutions were directly mixed with scintillation cocktail

1 2 TLC-scanning

Radioactive spots on TLC-plates were detected with a Desaga scanner (Type 12-2) equiped with a Berthold Zahlratenmesser LB 2031 and synchronously recorded

2 *Spectrophotometry*

2 1 Assay of diazepam and chlordiazepoxide for partition-coefficient determinations

Concentrations of diazepam and chlordiazepoxide in samples of each of the two solvents were measured after acidification of the aqueous sample or after extraction of the organic sample with 0 1 N HCl, on a Beckman DB-G grating spectrophotometer (diazepam λ_{max} 240 nm, chlordiazepoxide λ_{max} — 245 nm)

2 2 Assay of surfactant

Concentrations of polysorbate 80 and polysorbate 20 were measured on a Beckman DB-G grating spectrophotometer (λ_{max} — 232 nm)

Supernatant solutions appeared to contain less than 0 05 % surfactant

3 *Gas-liquid chromatography* (GLC)

3 1 Assay of meprobamate, carisoprodol and tybamate

Concentrations of meprobamate and its homologues were determined according to the gaschromatographic method of Douglas *et al* (1967)

Peak area were calculated as the product of height and width at half height

3.2. Assay of chlordiazepoxide, diazepam, oxydiazepam, desmethyldiazepam and oxazepam.

The diazepines were determined according to the gaschromatographic method indicated by DE SILVA *et al.* (1964) and described by MARCUCCI *et al.* (1968) and VAN DER KLEIJN (1969). Since chlordiazepoxide and oxydiazepam gave poor results when directly analyzed with gas-liquid chromatography, these compounds were hydrolyzed with 4 N HCl during 1 hr in a boiling-water-bath. After neutralizing the liquid with $NaHCO_3$, methylamino-5-chlor-benzophenone (MACB) and amino-chlor-benzophenone (ACB) were twice extracted with ether. The dried ether residues were taken up in 100 μl *n*-hexane and 5 μl were injected into the GLC.

4. *Thin-layer chromatography.*

Thin-layer chromatography (TLC) on precoated silicagel glassplates F 257 (Merck A.G., Darmstadt, Germany) was applied.

The following solvent systems were used:

1. Chloroform-acetone (9 : 1).
2. Chloroform-acetone-pyridine (75 : 25 : 5.6).
3. Ethylacetate-*n*-hexane-ammonia (12 %) (60 : 27 : 25).

Solvent 3 was used for checking radiopurity of the compounds. Solvents 1 and 2 were used to determine the migration-rate. Amounts of 5–10 μg were spotted on the plate and series of spots were simultaneously chromatographed at room-temperature over 10 cm. R_f values were measured in the usual way.

Biochemical procedures.

1. *Preparative ultracentrifugation.*

The experiments for the determination of binding to proteins and surfactants with ataractic drugs of the meprobamate and diazepine group were carried out in a Christ, Omega II 70 ultracentrifuge equiped with an auxiliary thermostatic set which allowed runs at 0°–40° C. At 37° C temperature equilibration was performed within $\pm$ 0.3° C. As a rule the rotor was started at about 43°–45° C and a stable temperature level was reached after about 20 min. Sedimentation was performed in a Ti-rotor 70 No 9798 carrying eight polycarbonate tubes ("Oak-Ridge" Type No 2854, contents $\pm$ 11 ml) closed with anticorrodal screw-caps of special design to withstand 420.000 g. Runs were made at 70,000 rpm, 37 $\pm$ 0.3° C for 3 hrs.

1.0 ml of supernatant fluid was taken off and analyzed for radioactivity or protein concentration.

The fraction of drug bound to protein or surfactant was calculated from the concentration in the supernatant and in the original solution after correction for protein volume.

In calculating binding parameters a correction has to be made for the relatively large volume displaced by the large macromolecules. According to MC LEAN and HASTING (1935) the apparent concentration values in protein-containing solutions were corrected by multiplying with a factor $100/P_w$ where:

$P_w = 99.6 - (0.75 \times \%$ protein) ml/100 ml plasma.

Corrections for polysorbate 80 solutions were applied as indicated by YAMADA and YAMAMOTO (1965) according to:

$P_w = 100 - (2.0 \times \%$ polysorbate) ml/100 ml solution.

2. *Determination of apparent molecular weight of nonpolar surfactants (micellar weight).*

The micellar weight of a nonpolar surfactant highly differs from the real molecular weight of the surfactant based on its chemical element composition.

Molecular weight of polysorbate 80 was determined according to ARCHIBALD's method using D_2O and H_2O (EDELSTEIN and SCHACHMAN, 1966, GROOT *et al*, 1969)

3 *Determination of protein concentration*

Total protein concentrations were determined with the biuret method

Protein spectra were densitometrically assayed after electrophoretical separation on cellulose-acetate using amido-black as dye.

4 *Assay of protein binding of meprobamate, carisoprodol, tybamate, diazepam and chlordiazepoxide "in vitro".*

4 1 Dog plasma.

Maximally 1 00 ml of aqueous solutions or suspensions of various concentrations were added to calibrated flasks, and the volume made up to 25 0 ml with outdated, heparinized dog plasma Radioactive drug corresponding to 1000–50 000 dpm/ml of final solution was introduced in a volume of 0 050 ml

Concentrated solutions of drugs, slightly soluble in water, were prepared by dissolving 1 part of drug in 1 part of ethanol (abs) and polysorbate 80, and by diluting with water to the desired volume The final polysorbate concentration in the stock suspension was 1 %

The pH of the solution was checked on a pH-meter, Radiometer Copenhagen type PHM 22 *r*, and was adjusted to 7 38

1 00 ml of plasma and supernatant fluid was assayed for radioactivity and protein concentrations

4 2 Bovine serum albumin (BSA)

10 % solutions of BSA in 0 25 M phosphate buffer and 0 25 M NaCl were made in stock 10 0 ml of stock solution was added to a calibrated flask of 25 0 ml

The drug was added as a 10 0 ml solution or a suspension as described above Various drug concentrations ranging from 50 ng — 200 μg ml were investigated

Radioactive material was introduced in a volume of 0 050 ml accounting for about 1000–50 000 dpm/ml of the final solution Volumes were made up to 25 0 ml with water

Concentrations of cold drug were considered to correspond with the weighed and diluted sample

4 3 Nonpolar surfactant

2, 5 and 10 % solutions of polysorbate 20 or polysorbate 80 were investigated. The drug concentrations used were similar to those of the BSA experiments Supernatant solutions were spectrophotometrically checked on polysorbate concentration

1 0 ml of the original solutions and the supernatants were assayed for radioactivity Results were correlated with the added drug concentrations

5 *Assay of protein binding of the ataractics in dogs "in vivo"*

Dogs, previously starved for 24 hrs, were injected intravenously with about 1–2 μC/kg body-weight corresponding to the doses given in Table I

TABLE I

Meprobamate	22–50 mg/kg
Carisoprodol	25–40 mg/kg
Tybamate	15–30 mg kg
Diazepam	0 9–2 mg kg
Chlordiazepoxide	1–4 mg/kg

Heparinized blood samples of 25 ml were taken from an implanted catheter in the arteria femoralis, and plasma and supernatant fluids were assayed for radioactivity.

Protein binding of the drugs was calculated from plasma and supernatant concentrations within 5 min after administration Biotransformation made accurate calculations impossible after 5 min (VAN DER KLEIJN, 1969[3]).

6. *Determination of partition coefficients.*

Determinations of partition coefficients were carried out by shaking equal volumes of a water phase, buffered to pH – 7.4 with 0 2 M phosphate buffer, and a with water immiscible organic phase. Chloroform, carbon tetrachloride, *n*-octanol, *n*-decanol and *n*-heptane were used as organic phases.

The compounds were solved in either the organic or the aqueous phase depending on their nature. Concentrations were determined either spectrophotometrically or gas-chromatographically When necessary aqueous phases were titrated with 0.1 N HCl. to the pH desired. The two phases were shaken for at least 24 hrs at room-temperature before analysis.

The partition coefficients in the *n*-heptane-water system correspond with the ratio's of the concentrations of the saturated solutions of the drug

RESULTS

Testing of the method.

The ultracentrifuge method was tested in order to determine the influence of sedimentation and temperature on protein binding.

The shortest possible sedimentation period was found to be 2 hrs. Fig. 1 and 2 show that a 2½ hrs run for dog plasma is sufficient to reach a protein-free supernatant of 2 ml. 3 hrs of centrifugation increased the security and was used throughout this study. The 5 hrs period resulted in the pattern as reported by SCHOLTAN (1965).

After the 2½ hrs period a plateau level at which drug and protein are in equilibrium could be distinguished in the centrifuge tube. At longer sedimentation periods the plateau level faded out (FIG. 1). In the BSA-experiments the lower protein concentration (4 %) resulted in a higher sedimentation rate and hence in a greater protein-free supernatant volume (FIG. 3).

The duration of the sedimentation period had no influence on the concentration of the drug in the protein-free supernatant solution (FIG. 1).

The influence of the temperature was studied by centrifugation at 37°, 20° and 15°C respectively. At lower temperatures higher protein-binding capacity was observed (FIG. 4). According to SCHOLTAN (1964) this phenomenon is more pronounced for substances with a low protein-binding capacity.

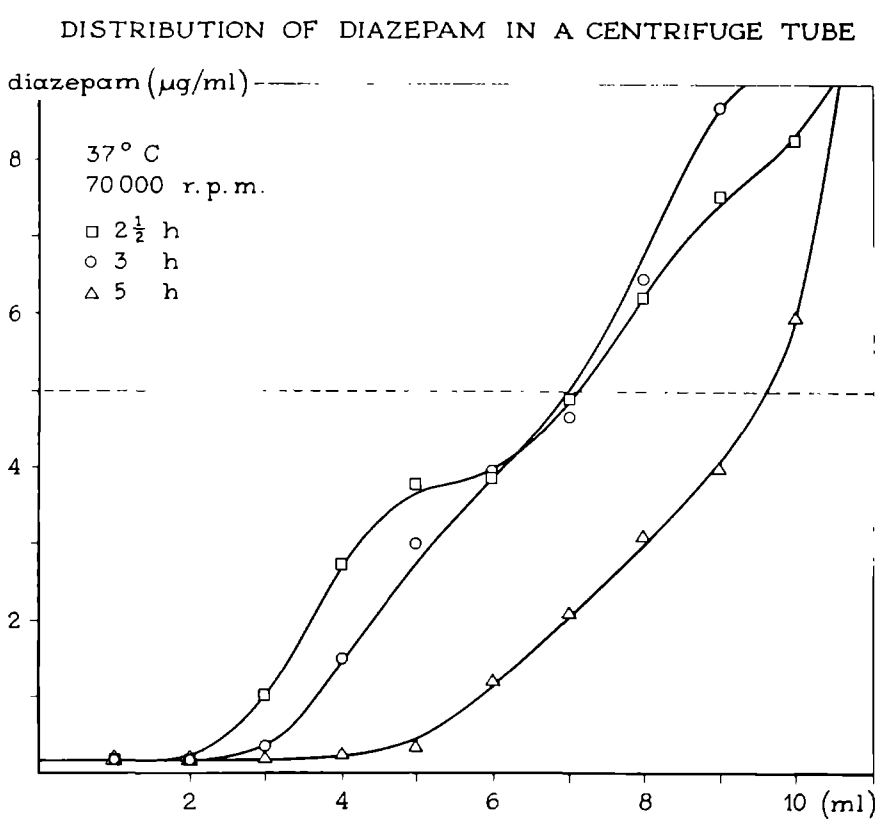

FIG. 1

Influence of sedimentation period on the volume of supernatant. 2½ hr of centrifugation of plasma might be sufficient to obtain a 2 ml protein-free supernatant.

Binding parameters.

For the estimation of the binding parameters the reciprocal plotting method of KLOTZ (1946) was used (r is the fraction of macromolecules occupied by drug molecules).

Binding of ataractic drugs to plasma proteins and nonpolar surfactants is summarized in Table II.

Fig. 5 and 6 indicate that the binding capacity shows little or no dependence on the total drug concentration in solution.

Figures 7 and 9 show the relation between $1/r$ and $1/[A_f]$. The reciprocal slopes are summarized in Table III. The ordinate intercept is difficult to determine. It appeared that this intercept which yields the value $1/n$ is considerably lower than 1.

TABLE II

Binding capacities of the ataractic drugs studied. The concentrations for which the values hold are given in figures 5 and 6

% Binding	Dog plasma ultracentrif. "*in vitro*" 37° C	Human plasma dialysis 20° C DOUGLAS e.a. (1964)	Polysorbate 80 ultracentrif. 5 %, 37° C	Polysorbate 20 ultracentrif. 5 %, 37° C	BSA ultracentrif. 4 %, 37° C	Dog plasma ultracentrif. "*in vivo*" 37° C
Meprobamate	0	0	50	42	38	8–13
Carisoprodol	45–47	55	73	66	46	41–44
Tybamate	63–71	77–81	81	78	59-65	54–70
Chlordiazepoxide	86–87		93	93	87	89.1–92.5
Diazepam	93–94		95	92	84–87	89.3–94.0

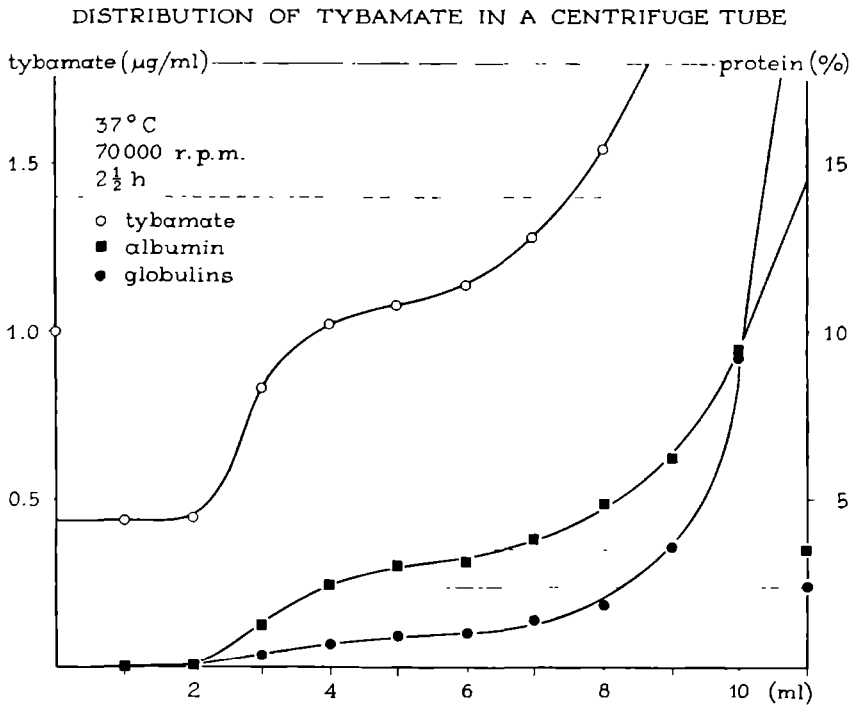

FIG. 2

Example of the distribution pattern of tybamate in a centrifuge tube after 2½ hr of centrifugation of dog plasma. The existence of a plateau level can vaguely be distinguished.

TABLE III

Binding parameters of ataractic drugs with BSA and nonpolar surfactants

Binding of ataractic drugs to BSA and nonpolar surfactants 37° C

Drug	$K_A \cdot n$ [10^3]		
	BSA	Polysorbate 80	Polysorbate 20
Meprobamate	1.15	1.56	1.04
Carisoprodol	1.41	1.56	2.48
Tybamate	2.82	2.67	4.32
Chlordiazepoxide	10.5	22	22.6
Diazepam	10.4	25	

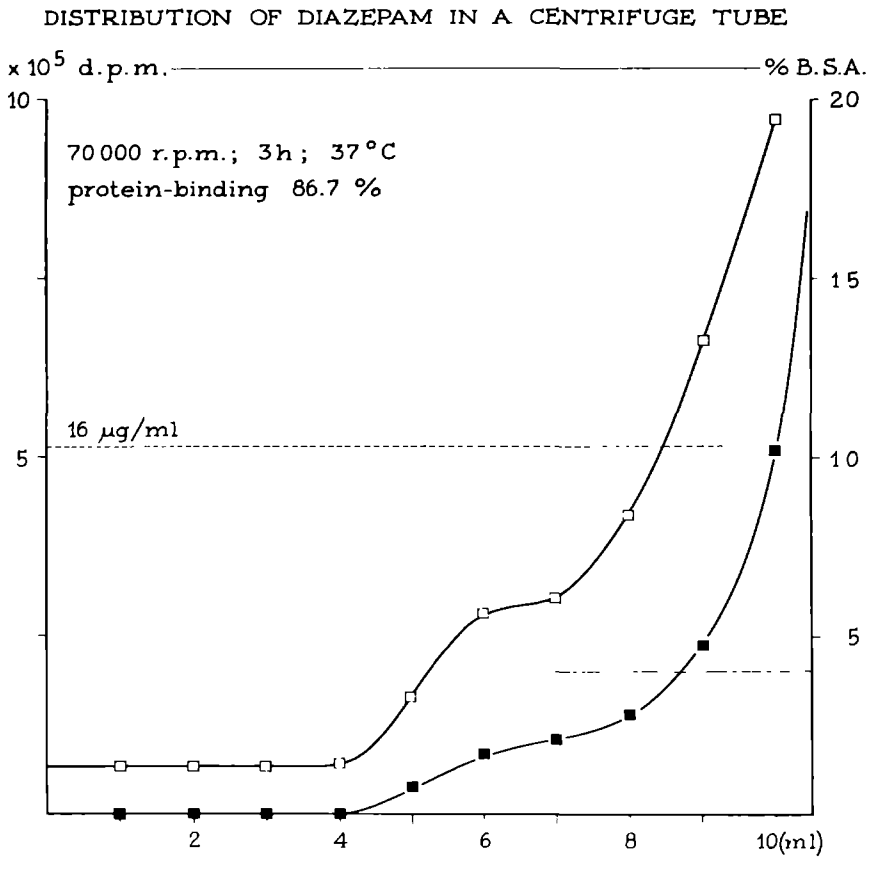

FIG. 3

Distribution of diazepam in a centrifuge tube after 3 hr centrifugation of BSA solution (4 %). Here a greater protein-free supernatant volume is achieved in the same period as in Figure 1.

TABLE IV

	conc. % w/v	λ_{max}	S_{20} value	M.W. micelle	M.W. (1) substance	d_{25} (1)
polysorbate 20	0.8	232 nm	1.3	60,000 (2)	1200	1.013
polysorbate 80	0.8	232 nm	1.6	74,400	1470	1.075

(1) Data of BECHER (1968).

(2) The micellar weight of polysorbate 20 has not yet been determined exactly; in calculations the value given was used.

This implies that more than 1 drug molecule binds to 1 protein molecule or micelle.

For the calculation of $1/r$ the molecular weight of the macromolecules has to be known. For BSA the generally accepted molecular weight of 69.000 was used.

Results of the estimations on the polysorbates are given in Table IV.

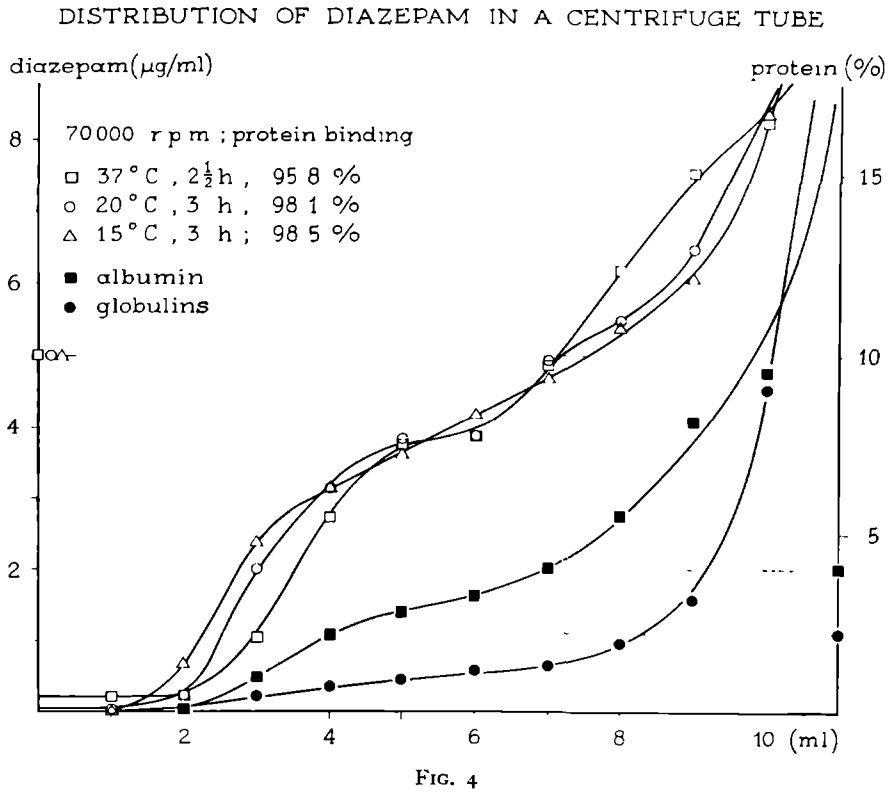

FIG. 4

Distribution patterns of diazepam in dog plasma.

Influence of temperature on sedimentation time. At lower temperature higher viscosity influences the sedimentation rate and subsequently the volume of the protein-free supernatant. Protein concentrations are given from the 37° C experiment.

Partition coefficients.

Determination of partition coefficients of the ataractic drugs was complicated by the extremely low solubility of the compounds in water and the high solubility in most organic solvents. Therefore differences in lipophilic character between diazepam, chlordiazepoxide and tybamate could only be established in the heptane-water-system (TABLE V).

True partition coefficient (TPC) values with other organic solvents are also given in Table V. TPC-values of diazepam and chlordiazepoxide were calculated from the apparent partition coefficient (APC) values (FIG. 10).

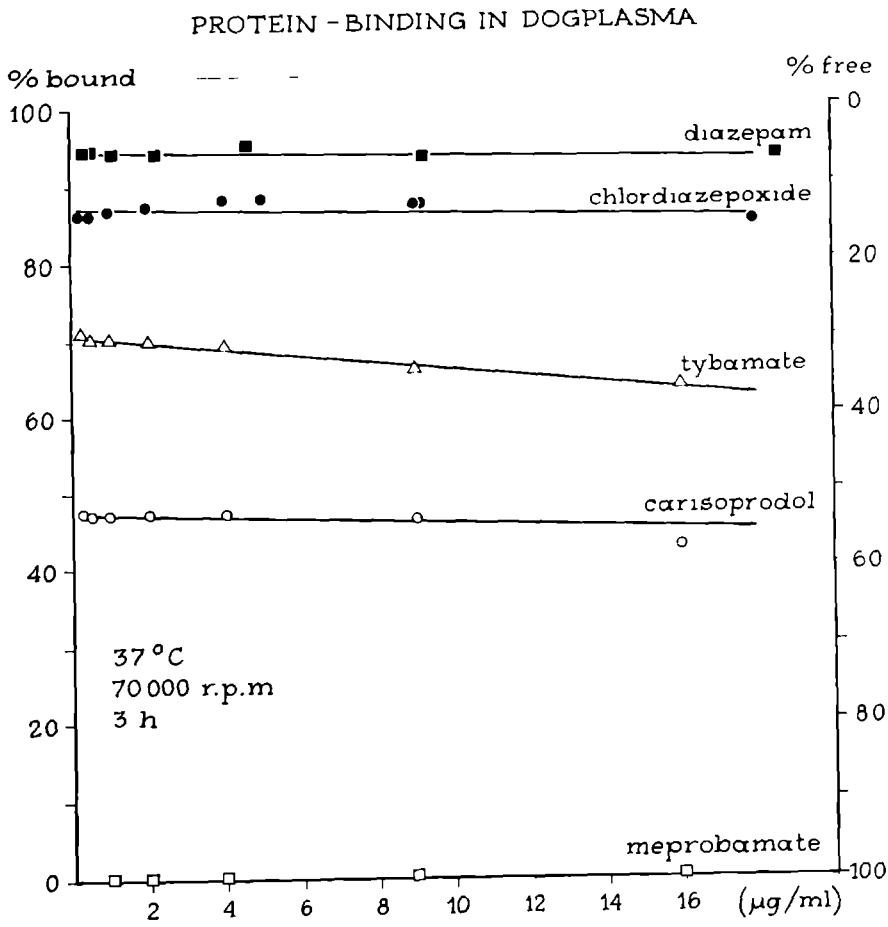

FIG. 5

Diagram demonstrating the protein-binding capacities of dog plasma for ataractics "*in vitro*" in the therapeutically achievable plasma concentration range The protein binding is not or only slightly dependent on the concentration

R_f-values.

Besides by partition coefficients and protein binding, lipophilic nature might be characterized by migration rates in chromatography represented by R_f-values. Table VI provides information about the relative lipophilic

character of the compounds. Thin-layer chromatography on silicagel plates in the given solvents appeared to give suitable data. Chlordiazepoxide is less lipophilic than diazepam, which correlates well with the rate of

TABLE V

Partition coefficients characterizing the lipophilic nature of drugs

Drug	True partition coefficient			
	Organic solvent / Water		pH – 7.4	23° C
	n-Heptane	*n*-Octanol	*n*-Decanol	Chloroform
Diazepam	150	180	280	1000
Oxydiazepam	1,4			
Desmethyldiazepam	0,6			
Tybamate	0.49	400	400	400
Chlordiazepoxide	0.2	130	170	550
Carisoprodol	0.04	80	400	400
Meprobamate	0.003	18	80	3.5
Oxazepam	< 0.01			

TABLE VI

R_f — values characterizing the lipophilic nature of drugs

Drug	R_f — value × 100	
	Solvent	
	1	2
Thiopentone	91	72
Hexobarbitone	82	67
Diazepam	76	64
Oxydiazepam	67	58
Tybamate	56	59
Carisoprodol	55	58
Phenobarbitone	52	57
Barbitone	51	56
Desmethyldiazepam	44	55
Phenazon	32	46
Oxazepam	24	39
Meprobamate	19	38
Chlordiazepoxide	19	35
Urea	2	3

penetration of the drugs into the brain (VAN DER KLEIJN, 1969). The same phenomenon was demonstrated for drugs of the barbiturate-series (CASSANO *et al.*, 1967).

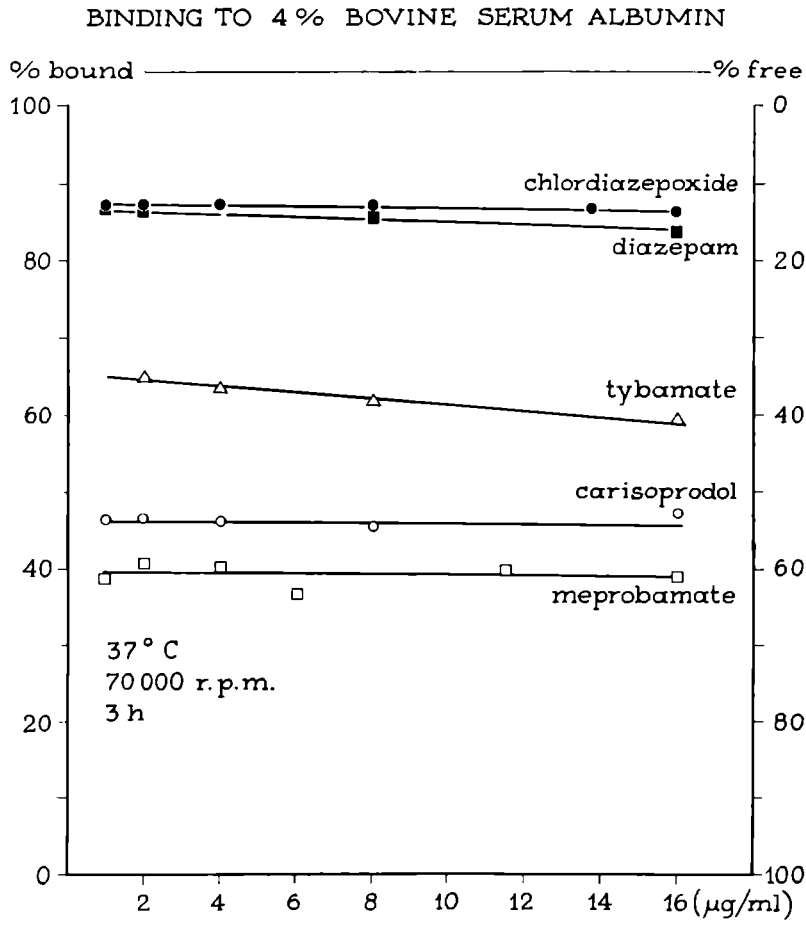

FIG. 6

Diagram demonstrating the binding capacities "*in vitro*" of BSA in the therapeutically achievable plasma concentration range of drugs and protein.

DISCUSSION

The main requirement of the methods for determination of protein binding is the imitation of the free, unbound, drug concentration in equilibrium with the drug-protein complex in physiological circum-

stances, since the free concentration is supposed to be responsible for diffusion to the biophase and hence for the pharmacological activity.

The classical and still most frequently applied method for the estimation of protein binding is the equilibrium dialysis technique (KLOTZ,

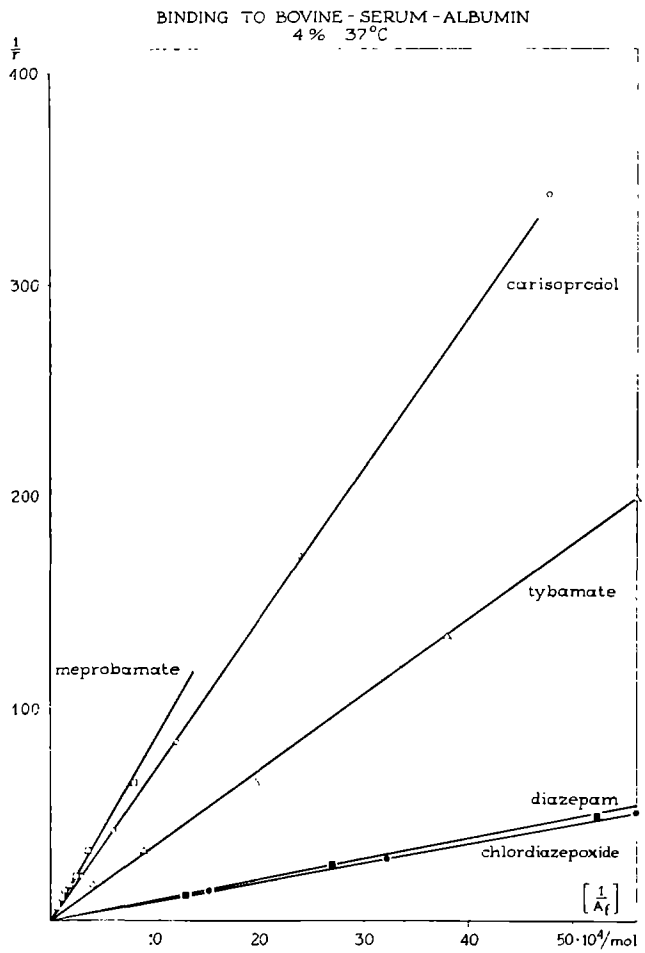

FIG. 7

Protein binding to BSA plotted according to KLOTZ (1946). Parameters are given in Table III.

1947; SPRING, 1966, and others). Although this method has been proved to give reliable results for other drugs, difficulties arose inherent to the materials and the equilibration time.

Other methods like ultrafiltration, electrophoresis (SCHOLTAN, 1962),

gel-filtration (KRIEGLSTEIN and KUSCHINSKY, 1968, 1969), analytical centrifugation (STEINBERG and SCHACHMAN, 1966), conductivity measurements and preparative ultracentrifugation (BÜTTNER and PORTWICH, 1961; SCHOLTAN, 1965) are used.

The preparative ultracentrifuge technique has been improved and appears to have certain advantages over other methods because of its fast and reproducible results, while absorption phenomena are lacking.

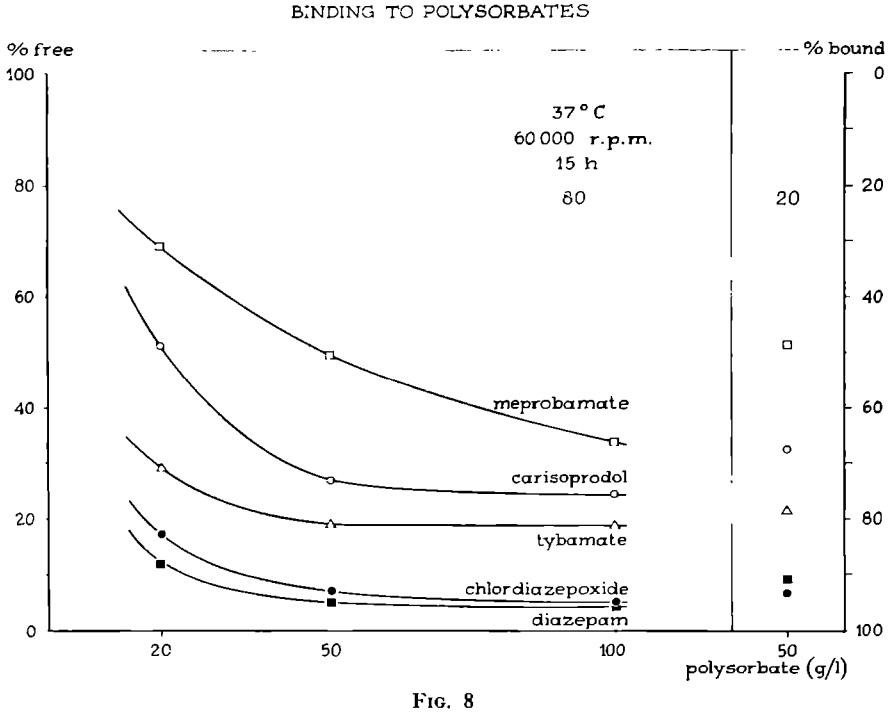

FIG. 8

Binding capacities of the polysorbates 80 and 20 for ataractic drugs.

SCHOLTAN (1965) found that the equilibrium situation during centrifugation is in accordance with the theory of GILBERT and JENKINS (1959). The synchronous sedimentation of drug-protein complex and protein as well as the existence of a level where all components are in equilibrium was confirmed. The latter phenomenon could only be achieved in a swinging-bucket rotor.

The sedimentation rate is a function of e.g. rotation rate and hence of the number of revolutions per minute to the second power, of temperature and concentration of solutes and hence of the viscosity (SVEDBERG, 1959). Thus the sedimentation time is inversely proportional to the

number of rpm to the second power. Increase in temperature and decrease in protein concentration result in a decrease in viscosity. The influence of the viscosity was not closely investigated. On the basis of the data of BÜTTNER and PORTWICH (1961) a run period of 5 hrs was calculated at 70 000 rpm.

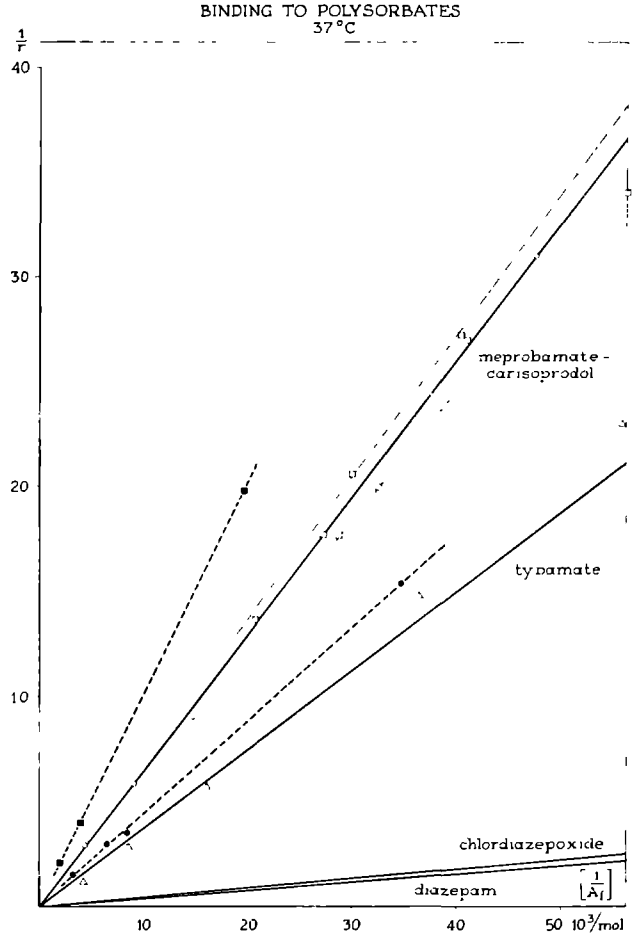

FIG. 9

Binding of ataractic drugs to polysorbate 80 and polysorbate 20. ———— 5 % polysorbate 80 solutions, ■ — — — ■ and ● – – – – ● 5 % polysorbate 20 solution, ..., — · — · — · — 2 % and 10 % polysorbate 80 solutions. The slopes are given in Table IV. Meprobamate □ and ■, carisoprodol ○ and ●, tybamate △.

Electrostatic equilibrium.

For solutions in which a mechanical separation takes place between

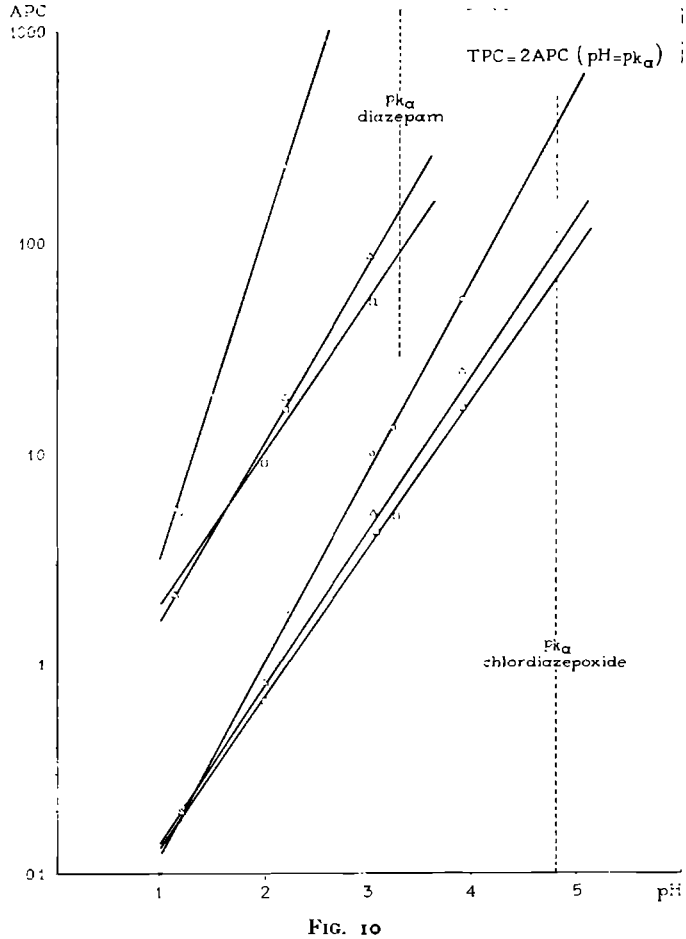

FIG. 10

Plot of APC against pH for calculation of the partition coefficients of the neutral form.
○——— ○ chloroform-water system.
△———△ *n*-decanol -water system.
□———□ *n*-octanol -water system.

charged particles of different molecular shape, corrections have to be considered.

In the ultracentrifuge technique the phenomena involved in this equilibrium can be distinguished as a primary and secondary salt effect (PEDERSEN, 1958).

The primary salt effect gives rise to reduced sedimentation of the charged macromolecule, because of a concentration gradient that is set up when the opposite charged particles have different sedimentation constants. As a result there will be an acceleration of the slow sedimenting

ions and a retardation on the colloidal ion since the condition of electrical neutrality must be fulfilled. Addition of neutral salts to the solution diminishes the potential gradient in a similar way as in the case of diffusion or of Donnan-equilibrium.

The secondary salt effect will occur even in more concentrated solutions when opposite charged ions set up an electrical gradient during centrifugation of unequally dense ions, e.g. CsCl. These effects have been neglected in our experiments.

Adsorption and polymerisation.

In equilibrium dialyse technique adsorption to the membrane has to be expected (RODRIGUES DE MIRANDA, 1967). Adsorption of radioactive material and polymerisation of the drug molecules leading to the formation of drug micelles might take place (JAENICKE, 1966).

In protein-free solutions no sedimentation under the same experimental circumstances could be established.

No adsorption on polycarbonate centrifuge tubes could be measured.

pH.

The influence of the pH on binding can be important under experimental conditions (GOLDBAUM and SMITH, 1954).

Meprobamate homologues are neutral compounds; diazepam and chlordiazepoxide are very weak bases (pK_a : 3.3 and 4.8 resp.), and are therefore practically neutral at the blood *p*H. In physiological circumstances, plasma *p*H variations are small and subsequent influence on protein binding is negligible.

Storage.

Bacterial contamination of the sample may change the binding capacity (SCHOLTAN, 1962). Several ways of preservation are investigated by KURZ and TRUNK (1968). Storage at 3-4°C for a period of 12 months had no influence on the binding capacity of plasma. Other methods, especially freeze-drying, were proved to be less favourable.

Treatment of the data.

Presentation of the percentages of drug bound and free drug is of little importance unless the concentration range is mentioned (GOLDSTEIN, 1949).

Drug-macromolecule interaction can be described as a reversible bimolecular reaction characterized by an association or a dissociation constant according to the mass-action law.

The fraction of total protein to which drug molecules are bound may be represented by the following equation (KLOTZ, 1946):

$$r = \frac{nK_A[A_F]}{1 + K_A[A_F]} \quad \text{or} \quad r = \frac{n[A_F]}{\frac{1}{K_A} + [A_F]}$$

This equation can be rearranged to

$$\frac{1}{r} = \frac{1}{n} + \frac{1}{nK_A} \cdot \frac{1}{[A_F]}$$

$r = \frac{[PA]}{[P_t]}$ = fraction of macromolecules occupied by drug molecules

n = number of independent binding places, demonstrating the same affinity, while no mutual interaction takes place.

K_A = association constant

$[A_F]$ = unbound-drug concentration

$[PA]$ = bound-drug concentration

$[P_t]$ = total protein concentration

When $1/r$ is plotted against $1/[A_F]$, n can be found as the reciprocal value of the ordinate intercept and K_A can be calculated from the slope ($1/nK_A$) (FIG. 7 and 9).

To include more classes of binding places, saturation of the first class must theoretically be fulfilled. In our experiments the solubility of the compounds was extremely low, which made experiments possible only in dilute solutions.

The binding of ataractic drugs to plasma proteins of dogs "*in vivo*" and "*in vitro*" and to BSA and surfactants "*in vitro*" appeared to show little dependence on the concentration over a wide range.

Similar findings have been reported for other drugs, for instance, heart glycosides by SCHOLTAN (1966) and KUSCHINSKY (1969), chlorpromazine and promazine by KRIEGLSTEIN and KUSCHINSKY (1968, 1969), and medazepam by RIEDER *et al.* (1969).

For all compounds used in this study, by reciprocal plotting according to KLOTZ a straight line was found (FIG. 7 and 9). No exact values for the amount of binding places (n) could be established since it was experimentally impossible to reach high enough drug concentrations, equimolar to protein or surfactant, because of the low water solubility of the compounds and because $1/n$ was found to be much lower than 1.

Plotting $-\frac{r}{[A]}$ versus r according to SCATCHARD (1949) gave no better information about n.

Similar experiments were carried out with nonpolar surfactants.

Nonpolar surfactants were chosen in order to exclude the contributions of possible electrostatic phenomena between the basic diazepins and the anionic sites at the protein molecule. Polysorbates have the advantages over plasmaproteins of greater stability and constant quality.

The close resemblance of the binding might suggest similar phenomena of solubilization by micelles that might also take place in blood. Both in protein and micelle binding, predominantly VAN DER WAALS forces must be considered (SCHOLTAN, 1964).

The binding by micelles may also be characterized as a partition between the water and the micellar phase, and subsequently micellar partition coefficients can be calculated (YAMADA and YAMAMOTO, 1965).

When a compound, solved in liquid, is brought in contact with another immiscible phase a constant concentration ratio will arise. This constant holds for the ideal case that the liquid phases are completely immiscible, that the solute cannot associate or dissociate in either of the two phases, and that the concentrations of the solutes are relatively low (REESE *et al.*, 1964).

The partition coefficient is in general an apparent partition coefficient (APC) which is defined as :

$$APC = C_1/C_2$$

C_1 and C_2 represent the concentrations of the solute in the organic (1) and in the aqueous (2) phase. To yield the true partition coefficient (TPC), being the quotient of the concentrations of the neutral form in both liquid phases (REESE *et al.* 1964), the APC has to be corrected.

For a base the well-known HENDERSON-HASSELBALCH equation can be read as :

$$TPC \cdot APC(1 + 10^{pk_a - pH})$$

The choice of the system to determine partition coefficients is of importance since it is often very difficult to collect drugs of highly different physicochemical properties into one system. Mineral oils (DOUGLAS *et al.*, 1964), chloroform, heptane (BRODIE, 1964), *n*-octanol (HANSCH *et al.*, 1967), oleyl alcohol (BUECHI and PERLIA, 1960) and systems of controlled polarity (FELTKAMP, 1965, and others) are used against water or buffer solutions.

Reversed thin-layer partition chromatography according to BUECHI and FRESEN (1966) appeared not to be useful for the highly lipophilic compounds.

Some of the compounds studied showed an extremely high lipid solubility which made direct measurements of TPC's inaccurate. APC's

of diazepam and of chlordiazepoxide were determined at different *p*H-values and TPC's were calculated from the APC's constructed at the *p*H equal to the pK_a (FIG. 10).

A close parallel between lipophilic nature and protein binding capacity was earlier reported by DOUGLAS *et al.* (1964) for the meprobamate-group and could be deduced from the data given by GOLDBAUM (1954) and FELTKAMP (1965) for barbiturates.

In the groups of ataractics used in this study lipophilic nature expressed as partition coefficients or R_f-values cannot similarly be compared to the binding parameters. Chordiazepoxide, which shows a higher protein-binding capacity, shows a lower partition coefficient and R_f-value than tybamate.

Diffusion coefficients measured through artificial membranes and permeability coefficients measured in artificial monolayers of membrane lipids can also be used to simulate the behaviour of drugs in biological systems (DOLUISIO and SWINTOSKY, 1964; KHALIL and MARTIN, 1967; GARRETT and CHEMBURKAR, 1968; VAN DEENEN and DEMEL, 1965).

DAVIS (1943) postulated that of chemotherapeutics that are partially bound to plasma proteins only the unbound fraction of the drug should participate in the chemotherapeutic action. Later this hypothesis was substantiated by many authors for many sulfonamides and antibiotics "*in vivo*" as well as "*in vitro*" (ANTON, 1961, SPRING, 1966). MARTIN (1965) calculated that compounds, having no tissue constituent binding, will only be restricted in their elimination from the blood by protein binding, if the association constant (K_A) is greater than 10^4.

Influence of protein binding on the distribution and elimination kinetics of drugs has been reported for sulfonamides, antibiotics and radiopaques when excretion is predominantly controlled by renal glomerular filtration (SIROTA *et al.*, 1950; KUNIN, 1962; KRÜGER-THIEMER, 1965, ENGELEN, 1968). However, this is not observed with the ataractics used in this investigation.

Meprobamate showed the lowest penetration rate into the central nervous system. However, diazepam which is highly bound to plasma proteins was rapidly eliminated from the blood and rapidly taken up by the brain and other organs and tissues (VAN DER KLEIJN, 1969, 1, 2).

It may be concluded that the influence of protein binding on the elimination of the drugs from the blood is compensated by the lipid solubility.

It has been suggested that the concentration of the unbound drug in plasma is directly correlated with the drug concentration in other body compartments and that protein binding will influence the apparent

distribution volume. Both plasma and plasmawater can be considered as separate compartments closely related by the binding parameters. The influence of these parameters on pharmacokinetics may be intricated when several binding places and classes are present (KRÜGER-THIEMER, 1964).

The concentration independance of the protein binding of ataractics enables the calculations of apparent distribution volumes on the basis of plasmawater concentration by multiplying the plasma-concentration with a simple term.

SUMMARY

The binding of meprobamate, carisoprodol, tybamate, chlordiazepoxide and diazepam to proteins and nonpolar surfactants was studied with the use of the preparative ultracentrifuge technique. Binding appeared to be independent of the drug concentration over the therapeutically possible plasma concentration range.

Protein-binding phenomena of the compounds are suggested to be nonspecific and comparable to those involved in solubilization by micelles.

Plasma protein binding is correlated with physicochemical properties like partition coefficients between two immiscible phases. In addition the significance of protein binding on the rate of penetration of ataractic drugs into the central nervous system is discussed.

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CHAPTER III

WHOLE BODY AUTORADIOGRAPHY

Application to water- and lipid-soluble and volatile compounds

INTRODUCTION

Since the introduction of contact autoradiography of whole animals in distribution studies of drugs, biogenous compounds, environmental pollutants, poisons etc., progressively increasing information has become available.

The most frequently used method today was first published by ULLBERG (1954) for ^{35}S-benzylpenicillin. Many radioactive elements have been tested for their utility (ULLBERG, 1961; FORBERG *et al.*, 1964). The photographic material is generally the limiting factor in the choice of the radiosource. ^{14}C-, ^{35}S- and ^{3}H-compounds are suitable for qualitatively good pictures.

The histological fixation by lyophilization enabled the permanent localization of drugs in organs and tissues not easily available for analysis, without postmortal diffusion and exchange or disturbance of the "*in vivo*" situation. This technique, however, is restricted to the application of non-volatile compounds.

In the research of drugs with highly specific action and localization after relatively low doses, this technique has many advantages when combined with the classical tissue extraction-separation procedures.

Quantitative analysis of the pictures has been difficult since densitometrical measurements suffer from inaccuracy.

The frequent simultaneous appearance of radioactive metabolites diminishes the value of the interpretations on the parent drug in the autoradiograms. Radiochemical separation techniques or selective labeling at a position of the drug which is primarily metabolized and excreted increases the value. When metabolism of drugs leads to the formation of volatile radioactive products, simultaneous application of the lyophilizing and non-lyophilizing procedures increases the information.

In this chapter the technique of the "whole body" autoradiography is described.

Test animals

Mice, rats, gerbils, foetus and organs of greater animals, fishes and other animals can be used The dimensions of the object are limited by the capacity of the microtome.

Materials.

Carboxymethylcellulose (medium viscosity), halothane (Fluothane® ICI), solid carbon dioxide, isopentane (technical grade), magic transparent Scotch tape No 810 (3 M Leiden, Holland) 3½ inch and 1 3/4 inch for twin- and solosectioning, respectively, double-coated Scotch tape No 666 3/4 inch (3 M Leiden, Holland), G 5 nuclear emulsion Gelform (Ciba Ilford, Amsterdam, Holland), Rontgen-film Structurix-D7 (Agfa-Gevaert, Rijswijk, Holland) and Mylar polyvinylchloride foil, 3 65 μ thick (Dupont, The Hague, Holland).

TABLE I

Survey of the most frequently used β -radiating isotopes

Isotope	Particle energy	Dose/kg body-weight	Photographic emulsion	Exposure time
^{3}H	0 018 Mev	5–20 mC	G 5 emulsion Structurix-D7	30–150 d
^{35}S	0.167 Mev	0.500–3 mC	Structurix-D7 Dentus Rapid X-ray film	5–60 d
^{14}C	0 155 Mev	100–500 μC	"	15–80 d

DOSAGE PROCEDURES

Preparation of dosage form.

Parenteral and enteral administration Water-soluble compounds are dissolved in physiological salt solution and buffered to pH 3–9 Water-insoluble compounds are dissolved in a suitable volatile organic solvent After addition of polysorbate 80 the solvent is evaporated and physiological salt solution is added to the desired volume Solutions and suspensions are prepared to be administered in 5–10 ml kg

Suppositories for rectal administration The drug is dissolved or suspended in the suppository base by melting or by means of a volatile solvent which is evaporated afterwards

Treatment of the animals.

Parenteral administration Intravenous injection is applied in the caudate vein. Intramuscular injection is applied in the hind leg or in the fish tail Other ways of parenteral administration are applied in the common way

Oral administration. Small laboratory animals receive the drug via an intragastric canule, so that solutions, suspensions or solids are administered directly into the stomach.

Rectal and topical administration. The drug is administered in 2 g suppository base/kg by means of a canule (diameter inside 0.8 mm). After administration the rectum is ligated under local anaesthesia. Topical administration can be performed according to KOLB *et al.* (1967).

The animals are separately isolated in gauze cages to prevent contamination of skin or mouth with radioactive material.

PROCEDURE

At chosen survival periods after administration of the labeled com-

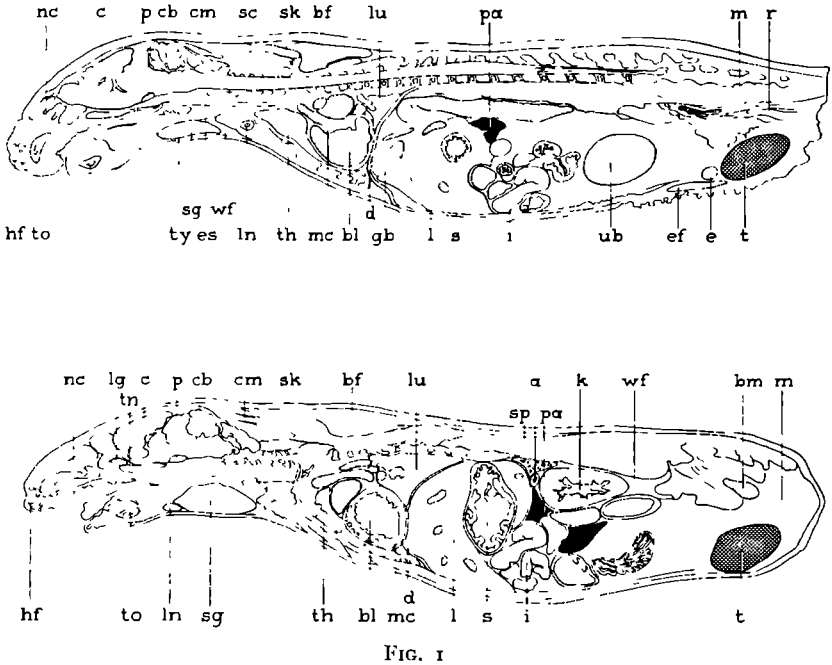

FIG. 1

Legend of the abbreviations.

A	adrenal gland	HF	hair follicle	R	rectum
BF	brown fat	I	intestine	S	stomach
BL	blood	K	kidney	SC	spinal cord
BM	bone marrow	L	liver	SG	salivary gland
C	cerebrum	LG	lachrymal gland	SK	skin
CB	cerebellum	LN	lymphe node	SP	spleen
CM	cervical muscle	LU	lung	T	testis
D	diaphragm	M	muscle (skeletal)	TH	thymus
E	epididymis	MC	myocardium	TN	trigeminal nerve
EF	epididymal fat	NC	nasal cavity	TO	tooth
ES	esophagus	P	pituitary gland	TY	thyroid
GB	gall bladder	PA	pancreas	UB	urinary bladder
				WF	white fat

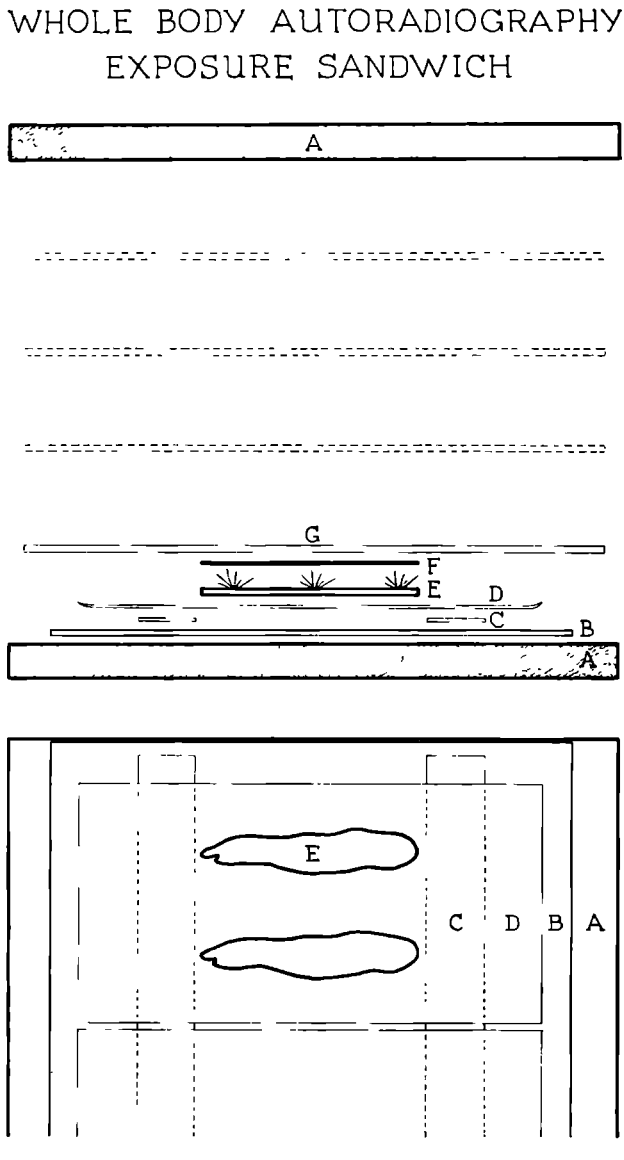

FIG. 2

Schematic representation of card-board for whole body autoradiography.
I. Water- and lipid-soluble compounds.
A. plank, B. card-board, C. double-coated Scotch tape, No. 666, D. Scotch tape No. 810, E. object, F. Röntgen-film, G. foamed plastic or filter paper.
II. Volatile compounds.
A, B, C, F, foil, E, D, G, ... A.

pound the animals are lightly anaesthetized with halothane. Before being sacrificed, the animals are stretched in forceps of special design to enable a reproducible position. The animals are killed by immersion into isopentane cooled with solid carbon dioxide to about -80°C. This method causes instantaneous death without asphyxia.

The animals are preserved on solid carbon dioxide before further processing. The objects are embedded in molds with a 5 %-solution of carboxy-methylcellulose, cooled to 0°C. The mass is cooled again by solid carbon dioxide and stored at -15°C.

Sagittal sections of 10-80 μ are made by a hydraulic driven Leitz sledge microtome, type 1300 or a Jung *K*-microtome, No. 171, type Ullberg, in a freezer at -15°C. The sections are made at several heights through the whole animal till all organs of interest are collected (FIG. 1).

The sections adhere to Scotch tape No. 810 during sectioning. The tapes with sections are transferred to card-boards (FIG. 2) and lyophilized in the freezer during 24 h. The card-boards are prepared with two parallel double-coated adhesive tapes (Scotch tape No. 666). Strips of Structurix-D7 film are pressed against the tapes and stored for exposure as long as necessary (^{35}S and ^{14}C). Tapes from sections with ^{3}H are adhered to glass-plates coated with *G* 5 emulsion. The whole procedure is carried out at -15°C. Films are removed at roomtemperature and developed according to common procedures.

Autoradiograms are selected and judged either directly (black area means presence of radioactivity) or enlarged on photographic paper (white area means presence of radioactivity).

Volatile compounds.

Compounds, volatile or liquid at roomtemperature, are handled according to the same procedure up to and including the slicing step. The histological sections are kept separate from the Rontgenfilm by Mylar foil (WIJHELS, 1969). Card-boards are handled in a different way. The sequence in the preparation of the card-board for volatile compounds is given by : A, B, C, F, foil, E, D, G, ... A. The lyophilization step is prevented. The slices are immediately brought in contact with the film and stored in a freezer at −80° C. The films are exposed four times longer than necessary in the previous procedure.

After four weeks no appreciable loss of water could be observed.

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CHAPTER IV

KINETICS OF DISTRIBUTION AND METABOLISM OF ATARACTICS OF THE MEPROBAMATE-GROUP IN MICE

INTRODUCTION

In the treatment of anxiety and psychic tensions, besides meprobamate, the N-isopropyl- and N-*n*-butylderivatives are used. Carisoprodol (Apesan®) and tybamate (Solacen®) respectively, show a number of common pharmacological properties with meprobamate.

The substituted derivatives are more lipophilic than the parent compound, as shown by partition coefficients and other physicochemical parameters (DOUGLAS *et al.*, 1964; VAN DER KLEIJN, 1968).

Differences in lipophilic nature have been correlated with pharmacokinetics of penetration into the central nervous system, of elimination and of metabolism (BRODIE *et al.*, 1960; CASSANO *et al.*, 1967; HESS *et al.*, 1968; VAN DER KLEIJN, 1969; KUNTZMAN *et al.*, 1967; GAUDETTE and BRODIE, 1959; LIEN and HANSCH, 1968). The distribution of tritiated meprobamate according to the Ullberg technique has been studied by EWALDSSON (1963). His findings could be confirmed with ^{14}C-meprobamate (VAN DER KLEIJN, 1967).

In this investigation the distribution of meprobamate, carisoprodol and tybamate, following different routes of administration at several survival periods was studied in male and pregnant female mice.

The first goal was to study the comparative distribution pattern of the three compounds and to interpret the patterns with those of the body volume indicators urea, phenazon, inulin and high molecular dextran.

The second goal was to correlate the results with the aid of the physicochemical properties of the compounds.

MATERIALS AND METHODS

Meprobamate-carbamate-^{14}C (spec. act.: 5.5 mC/mMole) was obtained from Volk, Radiochemical Company, Burbank, Calif., USA. Carisoprodol-(spec. act.: 1.96 mC/

mMole) and tybamate-carbamate-^{14}C (spec. act. : 6.2 mC/mMole) were generously supplied by Wallace Laboratories, Cranbury, N.Y., USA. The chemical and radiochemical purity of the compounds was checked by thin-layer chromatography (TLC) and by subsequent scanning with a Desaga-scanner type (12-2). All compounds did contain less than 1 % impurities. If necessary the compounds were purified by TLC. Bands corresponding to the parent compounds were scraped off and were prolonged eluted with dried aceton p.a.

Autoradiography.

Adult, male Swiss mice (average weight 20–22 g) and pregnant female mice two days before delivery (body-weight about 40 g) were used. The doses given are summarized in Table I :

TABLE I

Drug	Route of administration					
	Intravenous		Oral		Rectal	
Meprobamate	120 mg/kg	200 μc/kg	120 mg/kg	100 μc/kg	120 mg/kg	100 μc/kg
Carisoprodol	120	200			140	100
Tybamate	80	200			145	100

For intravenous (i.v) and oral administration the compounds were dissolved in dichlormethane containing polysorbate 80. The solvent was evaporated and the residue solubilized in 0.9 % NaCl-solution. The final polysorbate-concentration was 2 %. The mice received 0.01 ml/g body-weight. Injection was given in the caudate vein. Oral administration was performed by a canula into the stomach. For rectal administration the drugs were solved either in a mixture of macrogol 1500, macrogol 4000 (1 : 2) or in Estarinum B 2 g/kg was given in solid state by a canula (internal diameter 1 mm). After application the rectum was ligated under local anesthesia of lidocaine.

Immediately before sacrifice, the mice were lightly anesthetized with halothane, stretched, and rapidly frozen by immersion in isopentane, cooled to about −70° C with solid carbon dioxide. Stretching was performed in forceps of special design to improve the reproducebility of the sections of various animals.

Autoradiographic technique according to ULLBERG (1954, 1958) was used. The frozen animals were embedded in cooled carboxy methyl cellulose (5 %) solution, and were rapidly frozen again in solid carbon dioxide.

Sagittal sections, 30 μ thick, were made, simultaneously through two animals, in a refrigerated room (temperature about −12° C) with a hydraulic driven Leitz-sledge microtome 1300. After adhering to Scotch Tape (Permanent Mending Tape No. 810, 3 M Co., USA) the sections were freezedried and pressed on to Structurix (Gevaert) D 7 X-ray films and exposed for 15–40 days at −12° C.

Survival periods between 40 sec and 8 hours were chosen.

Tissue analysis of carisoprodol.

Male Swiss mice, previously fasted for 24 hours, were injected intravenously in the caudate vein with 50 mg (280 μC)/kg carisoprodol. They were killed 30 sec, 1, 3, 5,

10, 20, 40 and 90 min after administration, brains, hearts, lungs and livers were removed, and weighed The organs were homogenized with an Ultra-Turrax TP 18/2, in 2 vol of saturated KCl solution containing 0 1 % KCN The tubes and homogenizer were rinsed The decanted homogenates and rinsing fluids of each sample were collected and made up to 10 0 ml

1 0 ml of each homogenous fluid was transferred to a countvial and assayed for radioactivity

The remaining portion of the fluid was transferred into 40 ml tubes and extracted twice with 10 ml chloroform-carbontetrachloride (1 1), followed by two more extractions with 10 ml ethylacetate The organic phases were collected and evaporated

The residue was transferred quantitatively to thin-layer chromatography plates (TLC-plates) and developed

The remaining aqueous phase was concentrated in a countvial and assayed for total radioactivity

The efficiency of the extraction was calculated for each sample and accounted 80 99 % of the total-radioactivity, depending e g on tissue and survival period

Assay of radioactivity

Radioactivity of aqueous liquids was counted after mixing with 1 0 ml of 0 75 M Soluene (Packard) and subsequent warming for 45 min at 50° C After mixing with 15 0 ml of scintillation cocktail of BRAY (1960) the counting was performed with a Nuclear Chicago, Mark I, or a Packard Tricarb 3380 AAA liquid scintillation counter Corrections were made by the external standard method

Radioactive bands on TLC-plates were detected with a Desaga scanner (type 12-2) equipped with a Berthold Zahlratenmesser LB 2031, and were synchronously recorded

Quantitation of the scans was performed by estimating peaksurfaces as the product of peak height, and the width at half height

Spots and bands of intact carisoprodol were removed from the plates and suspended in 10 0 ml of a scintillation cocktail containing 4 g PPO, 0 250 g dimethyl-POPOP, 30 g Cab-O-Sil in 1 l toluene and assayed The efficiency of this procedure accounted 95 %.

Peak surfaces of the separated carisoprodol and meprobamate were correlated with the data obtained from liquid-scintillation-counting of the carisoprodol spots

Chromatography

Thin-layer-chromatography on precoated TLC-plates, Silica Gel F 254 (Merck A G , Darmstadt, Germany) and a solvent system containing ethylacetate-*n*-hexane-ammonia (12 %) (60 27 25) was used

RESULTS

General aspects of distribution of meprobamate, carisoprodol and tybamate

A uniform distribution pattern of meprobamate was already reported by EWALDSSON (1963) and VAN DER KLEIJN (1967) using ^{3}H- and ^{14}C-meprobamate respectively. In particular, at short periods after intravenous and oral administration it was noticed that brain, thymus and bodyfat showed the lowest concentration, compared to blood, lungs and skeletal muscles, while myocardium, liver, hypophysis and adrenal

cortex demonstrated a higher concentration. Fifteen minutes after administration the earlier observed differences were completely balanced out (FIG. 1). A very slow penetration of meprobamate into the central

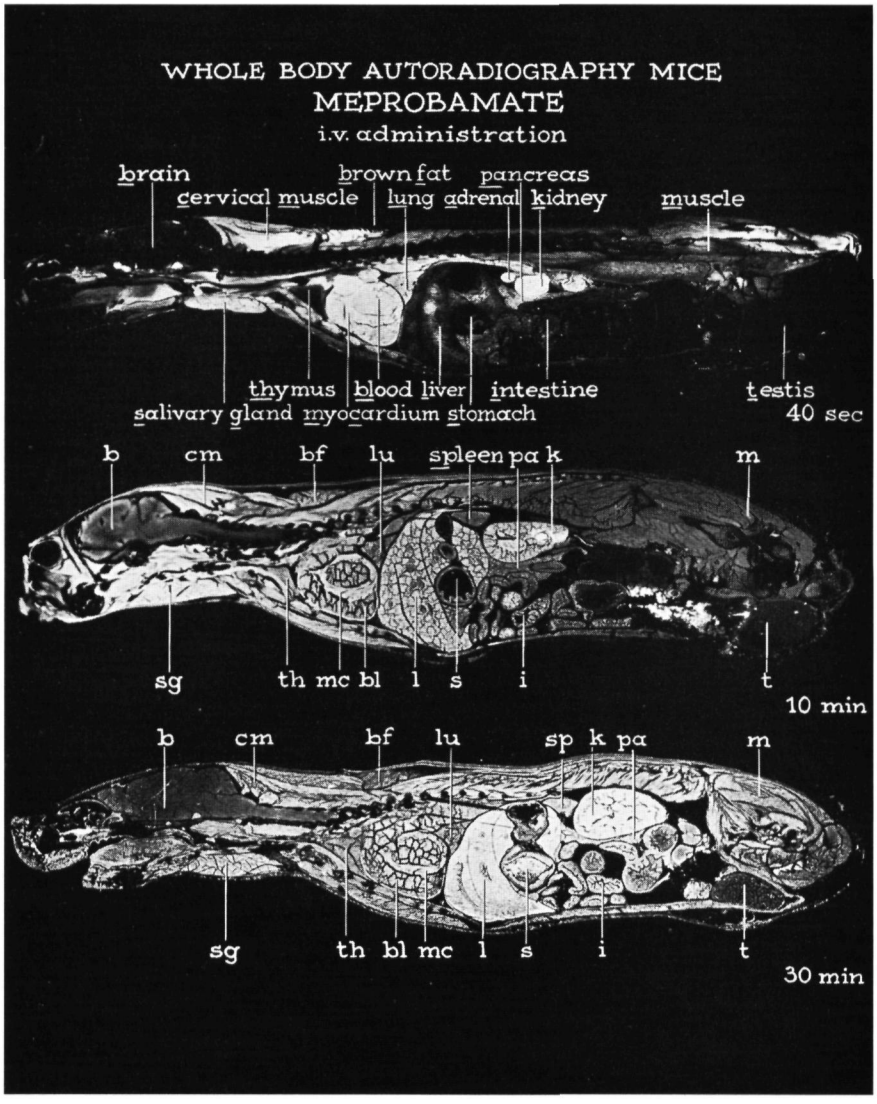

FIG. 1

Autoradiograms of mice after i.v. injection of ^{14}C-meprobamate. White area means presence of radioactivity. Notice the low concentration in the brain and the high concentration in blood, myocardium, kidney and cervical muscle at the 40 sec period, and the rather uniform distribution after 30 min.

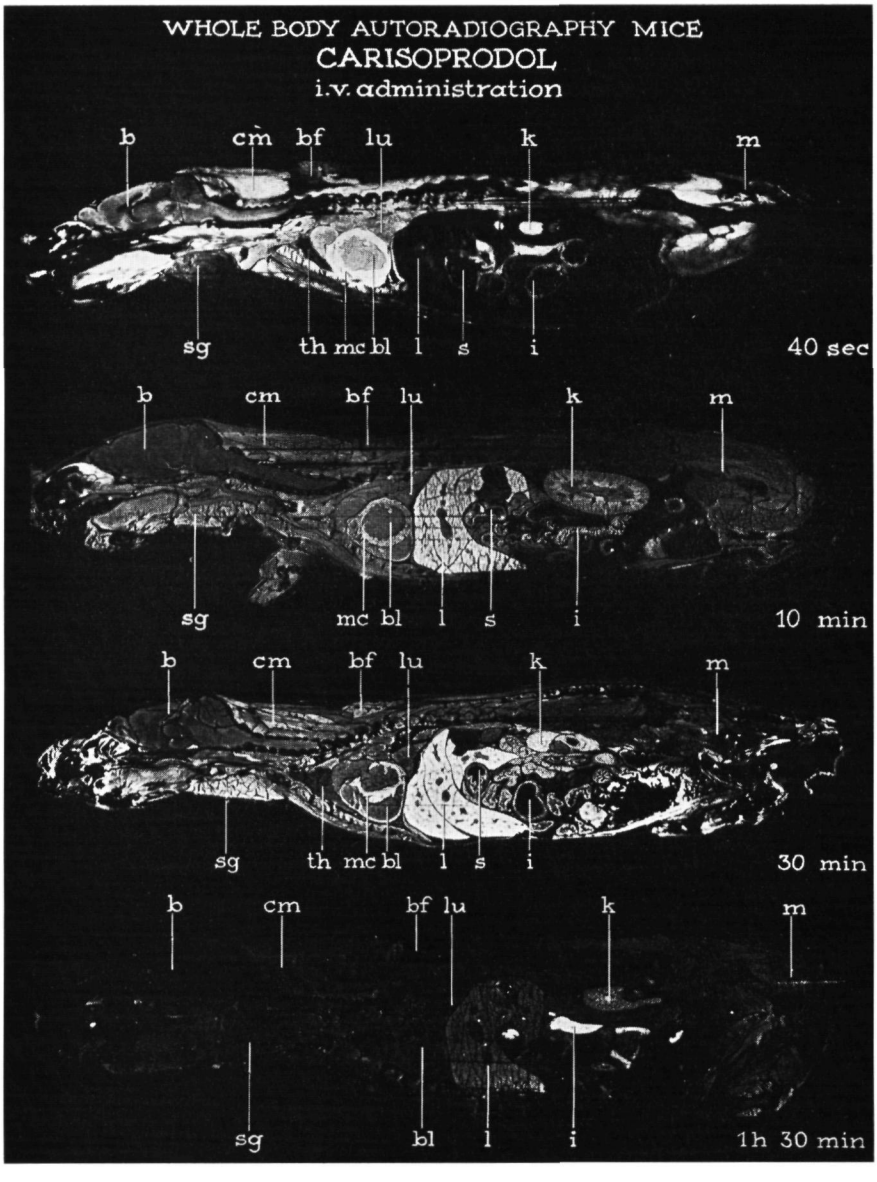

FIG. 2

Autoradiograms of mice after i.v. injection of ^{14}C-carisoprodol. White area means presence of radioactivity. Notice the high uptake of radioactivity in several regions of the brain 40 sec after administration and the close similarity with the pattern of meprobamate at the 10 min period.

nervous system was observed. The maximum concentration of radioactivity in the brain was reached 10-15 min after i.v. administration. However, carisoprodol and tybamate were rapidly taken up in the central

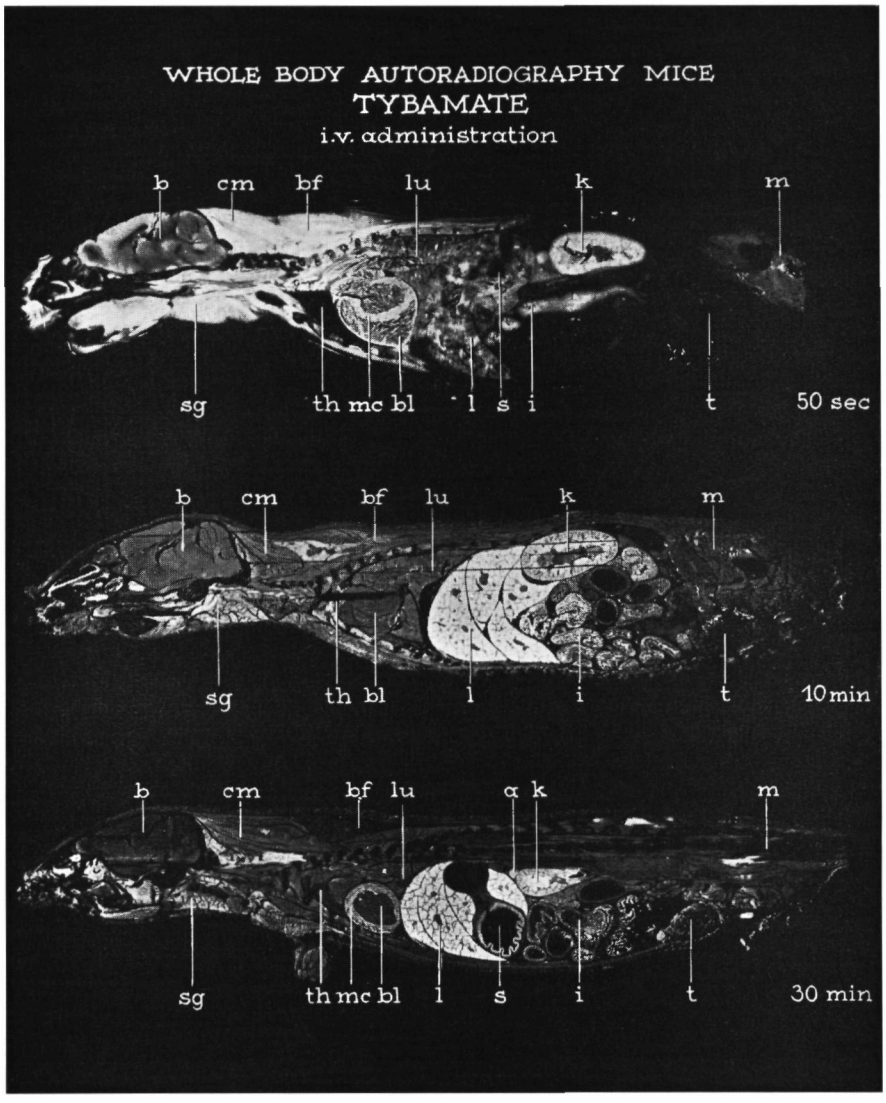

FIG. 3

Autoradiograms of mice after i.v. injection of ^{14}C-tybamate. White area means presence of radioactivity. Notice high uptake of radioactivity in several regions of the brain 50 sec after injection, and the close similarity with the pattern of meprobamate and carisoprodol at the 10 min period.

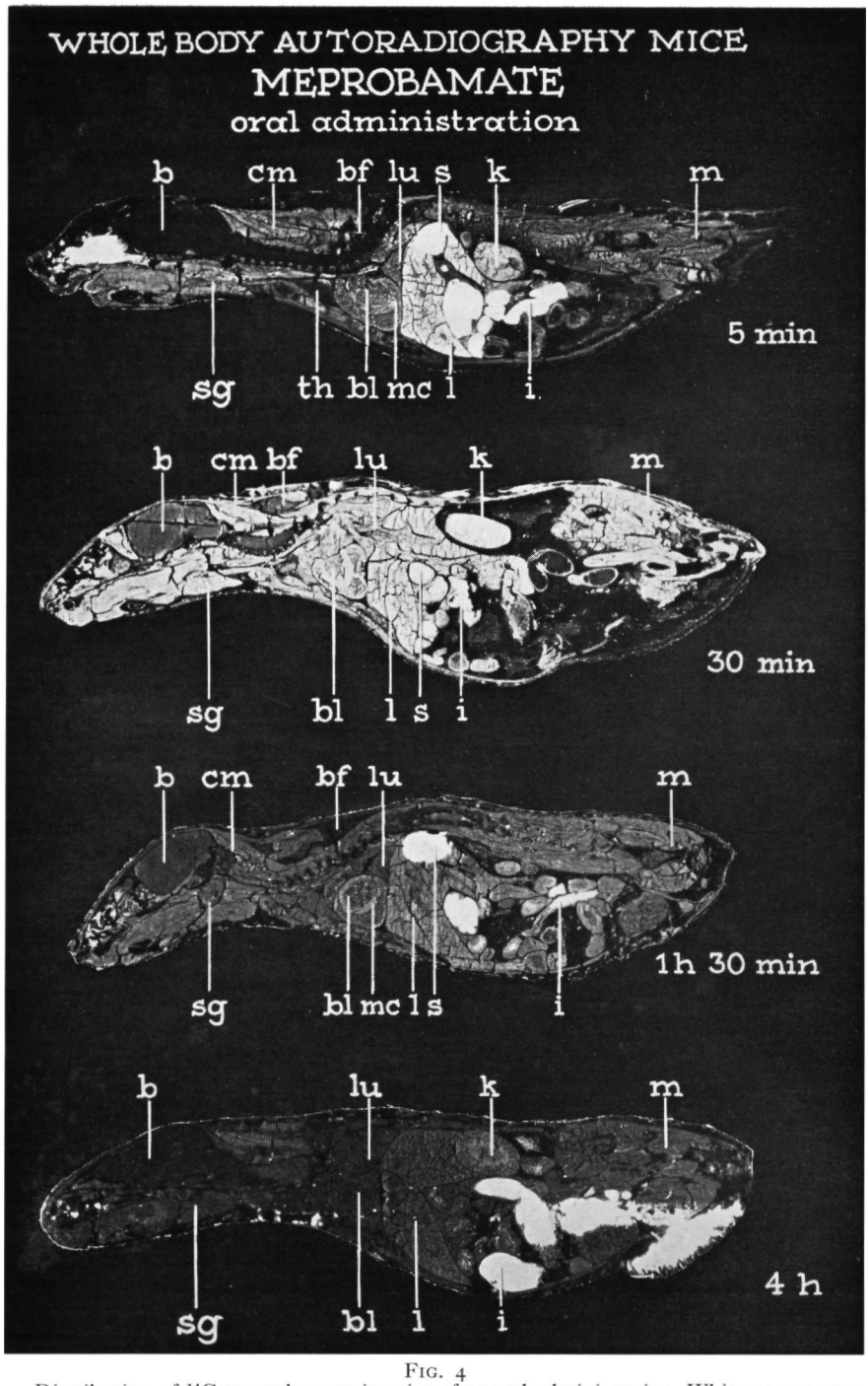

FIG. 4

Distribution of ^{14}C-meprobamate in mice after oral administration. White area means presence of radioactivity. Notice the rapid absorption from the gastro-intestinal tract 5 min after administration high concentrations are present in liver and kidney, but only low concentrations appear in the brain. After 30 min a rather uniform pattern can be observed. However, the brain shows a lower labeling than the blood, skeletal muscle etc.

nervous system. The concentration decay of radioactivity was very rapid as well, resulting in a similar distribution pattern such as meprobamate, reached about 5 min after i.v. administration (FIG. 2 and 3).

After rectal administration no differences in affinity of the central nervous system for the three compounds could be observed (FIG. 6).

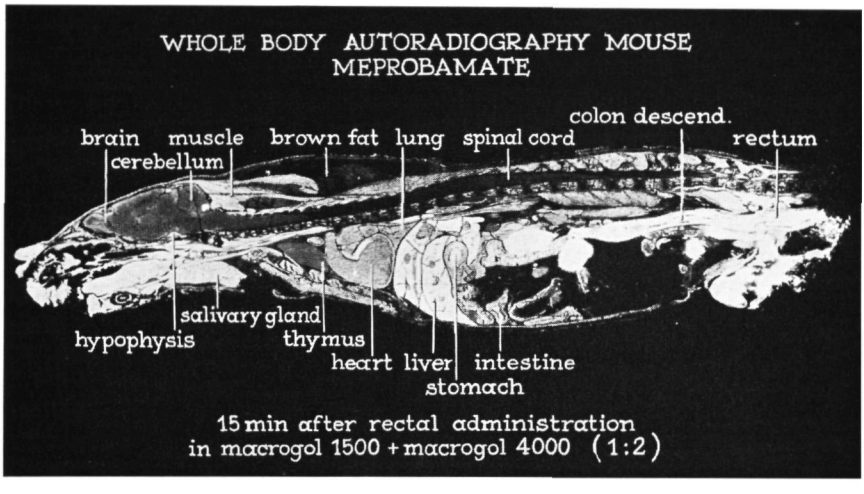

FIG. 5

Distribution of ^{14}C-meprobamate in a mouse 15 min after rectal administration. White area means presence of radioactivity. Notice the uniform distribution of radioactivity which is also demonstrated 15–30 min after i.v. and oral administration of meprobamate.

Specific aspects of distribution of meprobamate, carisoprodol and tybamate. Central nervous system.

The initial uptake of radioactive material differs greatly depending on the type of drug. A surprisingly low level of radioactivity was demonstrated in the brain until 10 min after i.v. administration of meprobamate. Carisoprodol and tybamate were very rapidly taken up by the strongly vascularized gray matter of cerebral and cerebellar cortex. Up to 5 min after injection high concentrations were found in cortex, hippocampus, thalamus, inferior colliculi and spinal cord. After 5 min differences in the anatomical regions of the brain were leveled out. Distinct from ataractic drugs of the diazepine-group no retention of radioactivity in the white matter of the CNS and in the peripheral nerves could be demonstrated after longer survival periods [VAN DER KLEIJN, 1969 (1)].

The maximum concentration of meprobamate was reached 15 min

after injection. Slight differences between the various regions of the brain could be observed, but were soon balanced out.

No variations could be distinguished anymore in the distribution of the radioactivity in the brain between the three different compounds 15 min after i.v. administration. The concentration of radioactivity at later survival periods was slightly lower than in the blood (FIG. 1, 2, 3, 8).

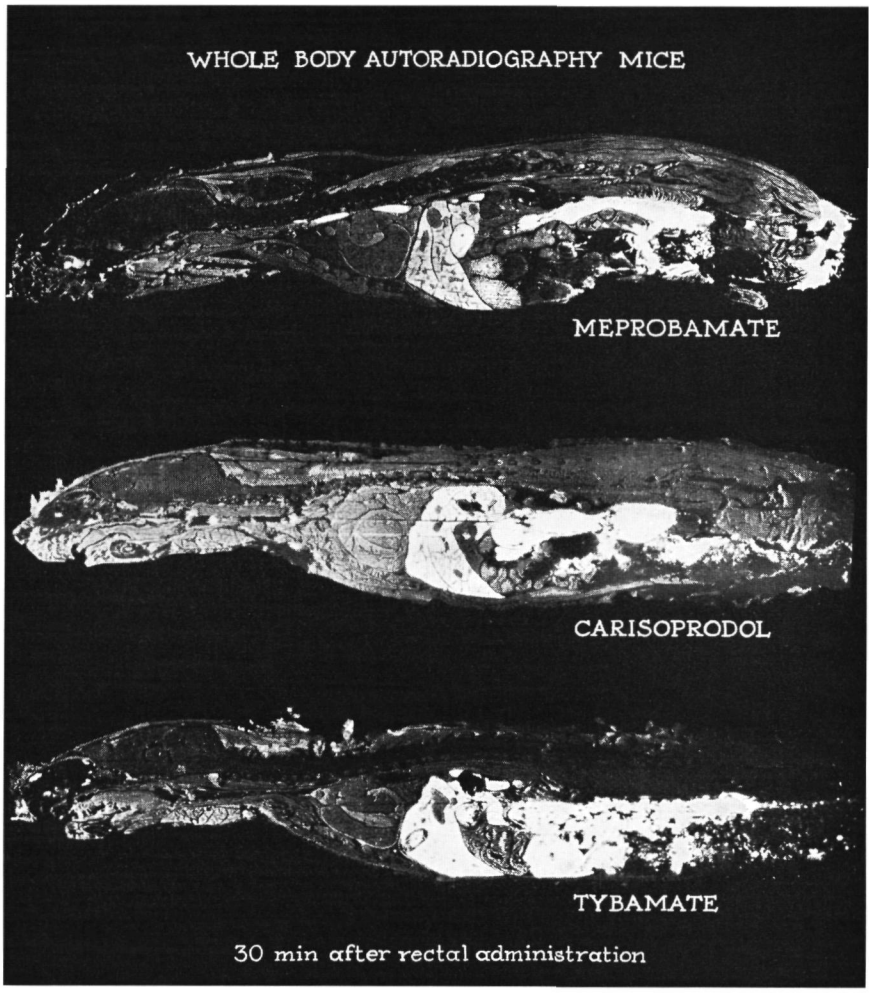

FIG. 6

Distribution of ^{14}C-meprobamate, ^{14}C-carisoprodol and ^{14}C-tybamate after rectal administration. White area means presence of radioactivity. No marked differences in general distribution can be observed. Only the concentration of radioactivity in the liver is higher after carisoprodol and tybamate treatment.

Cardiovascular system.

The radioactive compounds were very rapidly taken up in the myocardium. The labeling of the heart muscle rapidly decreases during the first 10-15 min after i.v. administration, but a slightly higher concentration than in the blood continued to be evident at all survival periods.

The concentration of the compounds in the blood was high at the early periods, but fell rapidly during the first 10 min after i.v. injection. Qualitatively the concentration in the blood became equal to the concentrations in skeletal muscle, lung, brown fat, intestine, salivary gland and other organs.

In the spleen no differences with the concentration in the blood nor between red and white pulp could be distinguished at all survival periods.

Liver.

The concentration of radioactive material accumulated during the first 5 min after injection. At the 10 min period an incomplete circulation could be demonstrated in the pictures. The concentration of radioactivity in the liver was the highest of all organs.

After 30 min accumulation of radioactive material in the bile and subsequent labeling of the lumen of the intestine could be demonstrated.

Digestive system.

At the early survival periods radioactivity rapidly appeared in the mucosal part of stomach and intestine. However, no excretion into the lumen could be noticed.

After 10 min a rather uniform distribution was observed over the whole gastrointestinal tract-wall.

Eye.

The eye rapidly accumulated the labeled material. Radioactivity due to the drugs was present in the choroidal layer. The decrease of radioactivity paralleled that of the blood.

A high amount of radioactivity was present in the lachrymal glands at all survival periods.

Endocrine glands.

The hypophysis built up a high level of radioactivity immediately after i.v. administration and retained a higher level compared to the brain at all survival periods.

The adrenal cortex contained a higher amount of labeled material than the medulla at all survival periods.

Urogenital system.

The radioactive drugs were rapidly taken up by the ovaries after i.v. administration. The corpora lutea showed a higher amount of labeled material compared to the surrounding tissue.

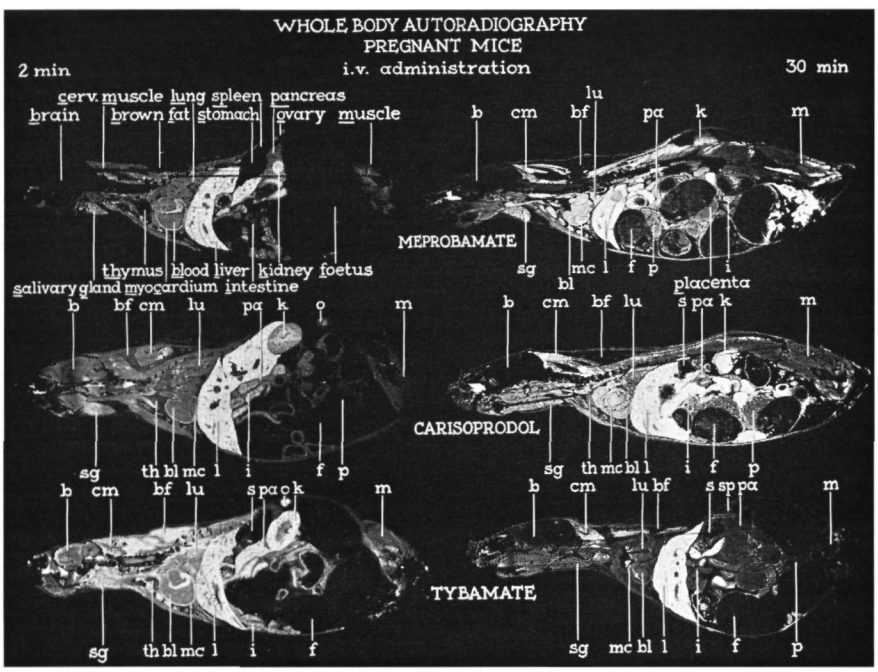

FIG. 7

Distribution of meprobamate-, carisoprodol- and tybamate-^{14}C in pregnant mice. White area means presence of radioactivity. Notice the similar distribution of radioactivity in the foetus 30 min after injection in spite of the different initial patterns in the mother.

The distribution pattern in the sexual organs of the male was rather uniform at all survival periods; labeled material appeared to be present in the interstitial tissue of the testis.

The kidney showed a high labeling within 40 sec after administration.

The concentration of radioactivity decreased parallel to that in the blood. After 10 min radioactivity appeared in the urinary bladder.

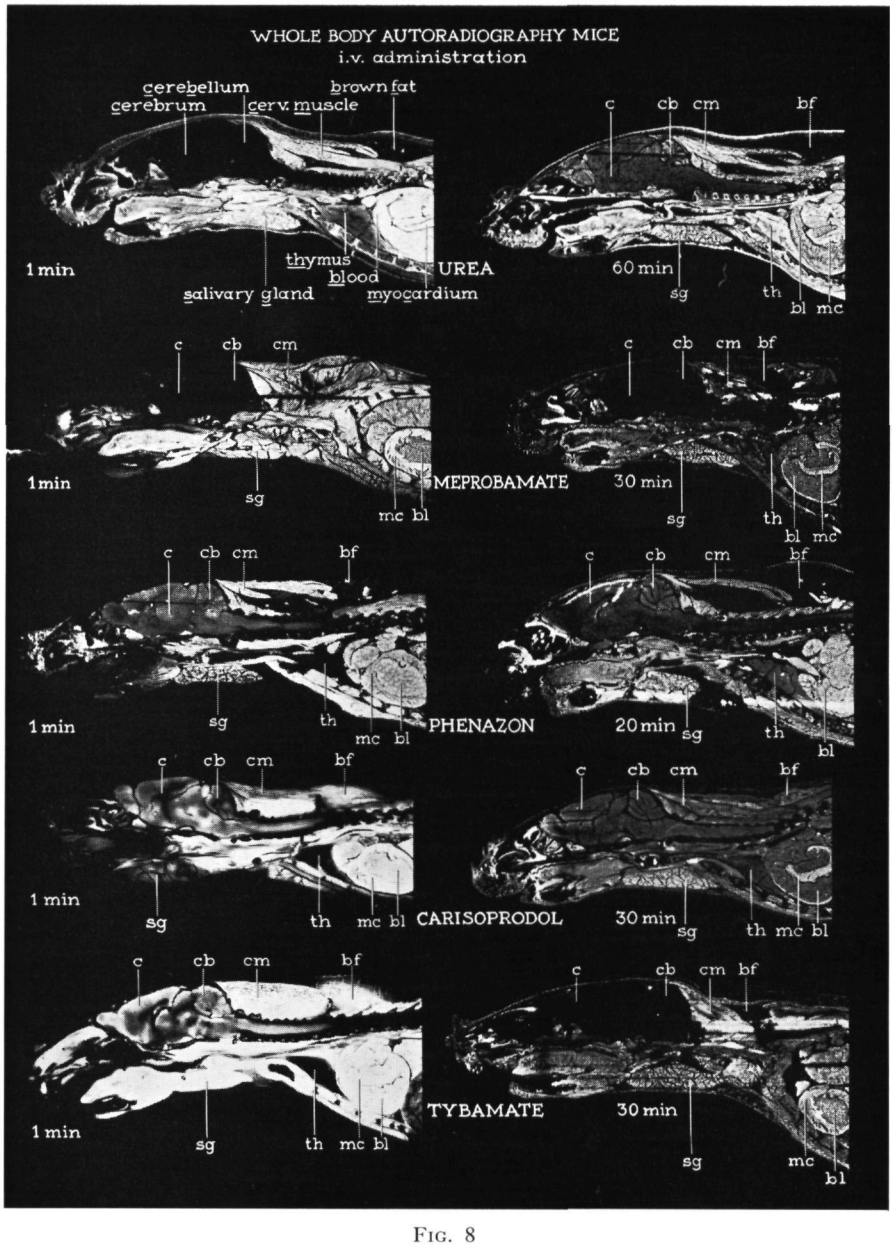

FIG. 8

Details of distribution patterns of urea-, meprobamate-, phenazon-, carisoprodol- and tybamate-^{14}C after intravenous administration. Notice the differences in uptake of the drugs in the central nervous system at the early survival periods. Although the periods, after which maximum concentration in the brain is reached, widely differ a great resemblance of the patterns of radioactivity is observed, when distribution equilibrium is achieved.

Lungs; lymphatic tissue; skeletal muscle; skin.

The radioactivity in the lungs, lymphatic tissue, skeletal muscle and skin did not differ from that in the blood during all survival periods. The uptake in the thymus was retarded compared to the other organs but after 5 min no differences could be observed. At the early survival periods the cervical muscle was rather strongly labeled as compared to other muscles.

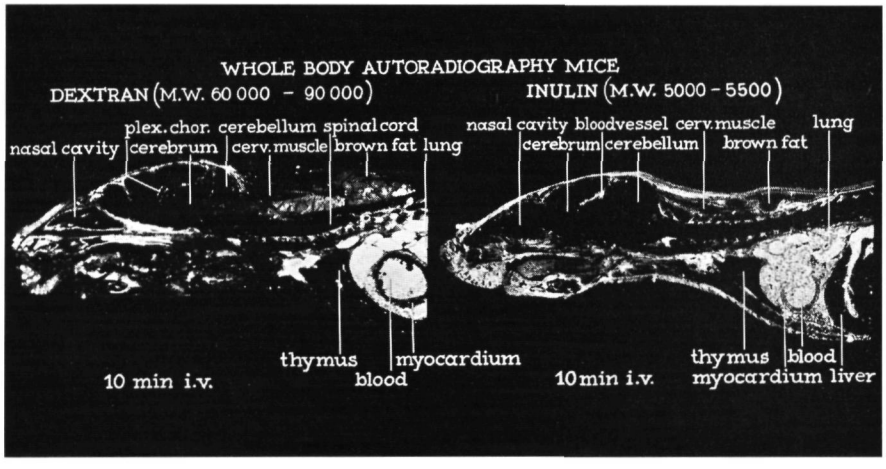

FIG. 9

Details of distribution patterns of inulin- and highmolecular-dextran-^{14}C after i.v. administration. Notice the differences in uptake of radioactivity by skeletal muscle, glandular tissue and bodyfat. The distribution of dextran is restricted to the intravasal space.

Adipose tissue.

In contrast to meprobamate, carisoprodol and tybamate were rather rapidly taken up in adipose tissue, especially in the brown fat. After 10 min the concentrations had been decreased to the level of blood and other tissues.

Distribution of meprobamate, carisoprodol and tybamate in pregnant mice.

The passage through the placenta was rather slow, 2 min after injection of either of the three compounds hardly any radioactivity could be detected in the foetus. After 15 min a uniform distribution of radioactivity was present in the foetus at a level slightly lower than in the blood of the mother.

After 30 min hardly any difference could be detected between the foetus and the mother. No differences were observed between the distri-

bution patterns of radioactivity derived from the three different compounds in the foetus (FIG. 7).

Distribution of meprobamate after oral administration.

Meprobamate was rapidly absorbed from the gastrointestinal tract following oral administration. 5 min after ingestion the distribution of the absorbed part of the dose was already rather uniform. Only very small

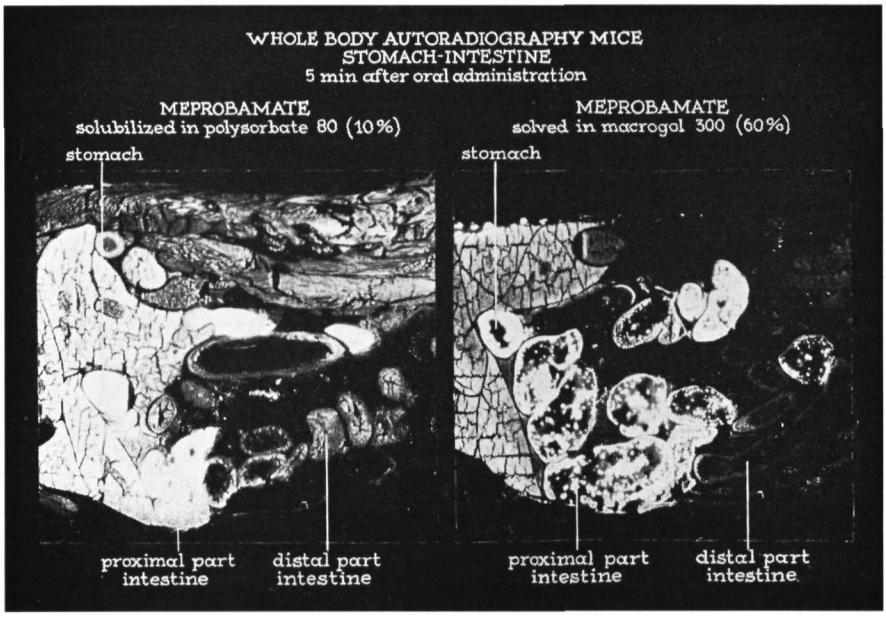

FIG. 10

Distribution patterns of meprobamate in the gastro-intestinal tract after administration in two different drug additives. Notice the swelling of the intestine caused by macrogol 300.

amounts were present in the brain. Obviously the gastrointestinal tract showed the highest amount of radioactivity. The liver showed a high affinity for the drug after 30 min. Moreover at longer survival periods the uniform pattern, characteristic for distribution over total body water, could be demonstrated (FIG. 4).

Since polysorbates were reported to have a retarding effect on absorption of drugs (LEVY and REUNING, 1964) correlated to micelle-binding properties, the meprobamate was orally administered either solubilized in 10 % polysorbate 80 (Tween 80) or solved in 60 % macrogol 300 (polyethyleneglycol 300). Autoradiograms, however, did not

show any correlation to the micelle-binding. Absorption of meprobamate appeared to be retarded when administered in macrogol 300 solution.

Evidence is provided that binding to the solubilizing substance is of minor importance compared with the action of the additive itself on the gastro-intestinal tract. Experiments on the isolated guinea-pig ileum showed a papaverine-like spasmolytic action of polysorbate 80 and macrogol 300 in concentrations which could be expected after oral administration. Macrogol 300 is a hygroscopic substance; in mice it

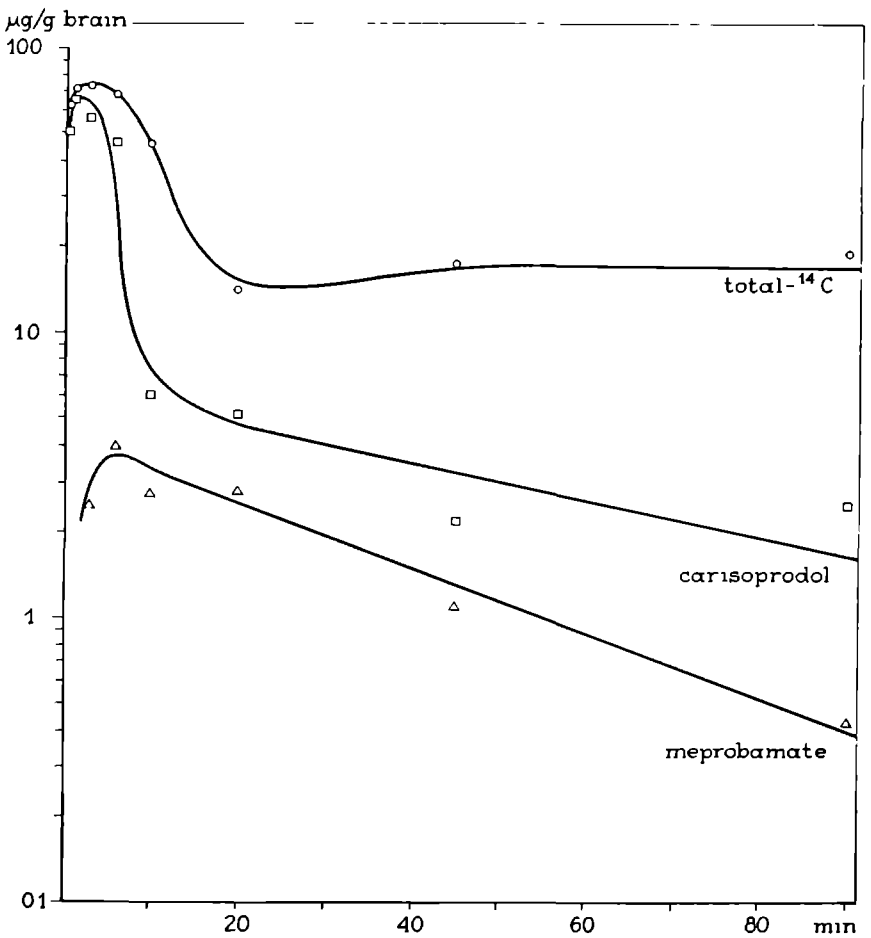

FIG. 11

Concentration decay curve of carisoprodol in brain of mice. Notice the rapid initial elimination of carisoprodol and total radioactivity. Meprobamate-formation is the major metabolic pathway in mice. The penetration of meprobamate into the brain is slow as compared with the penetration in other tissue. Compare Figure 1.

caused a swelling of the lumen of the intestine during its passage and a pronounced diarrhoea (FIG. 10).

Distribution of meprobamate, carisoprodol and tybamate after rectal administration.

Meprobamate was administered either solved in a macrogol 4000, macrogol 1500 mixture (2 : 1) or mixed with Estarinum B. The absorption appeared to be faster from the macrogol medium than from the fat medium (FIG. 5).

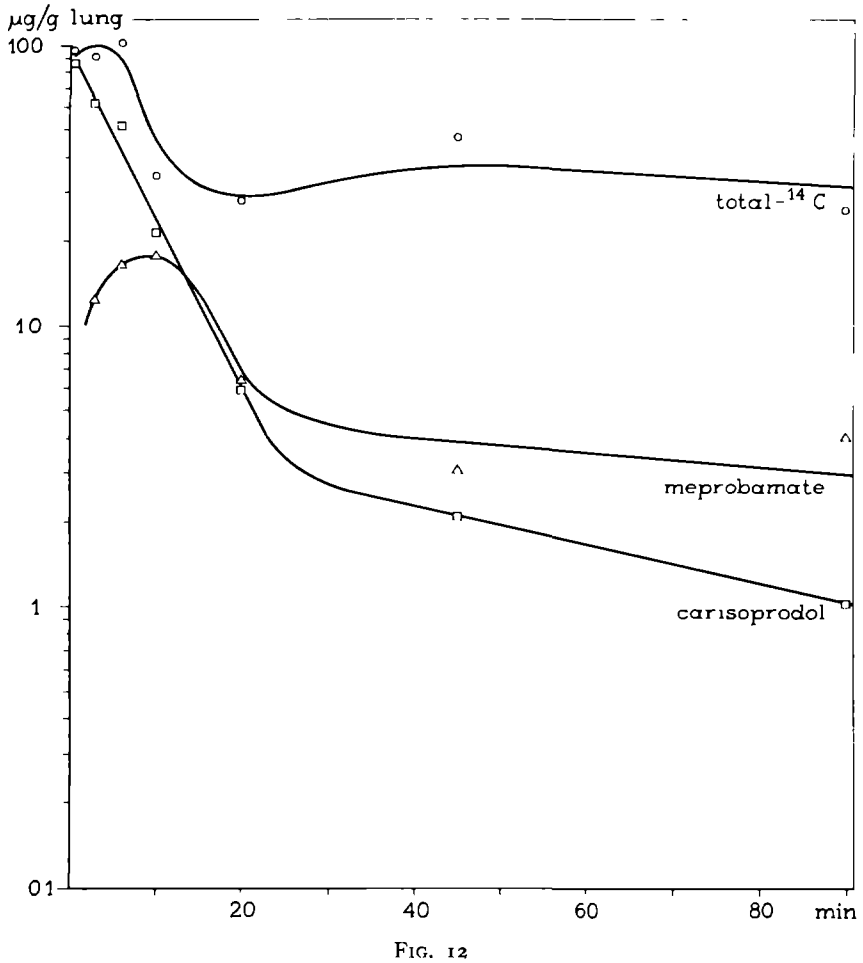

FIG. 12

Concentration decay curve of carisoprodol-^{14}C in lung of mice. According to the autoradiograms, concentration of radioactivity in lung is comparable with the concentration in blood. Notice the biphasic elimination pattern of carisoprodol. Meprobamate rapidly appears in the tissue.

No differences in the general distribution pattern between either meprobamate, carisoprodol or tybamate could be established (FIG. 6). The maximum concentrations were reached about 30 min after application. No special affinity of the radioactive material for the brain could be demonstrated in this way. The absorption and dissolution rate of the drugs are limiting factors in the distribution of the substituted drugs since dealkylation is a very fast process in mice. The general distribution pattern of radioactive material 30 min after rectal administration was in

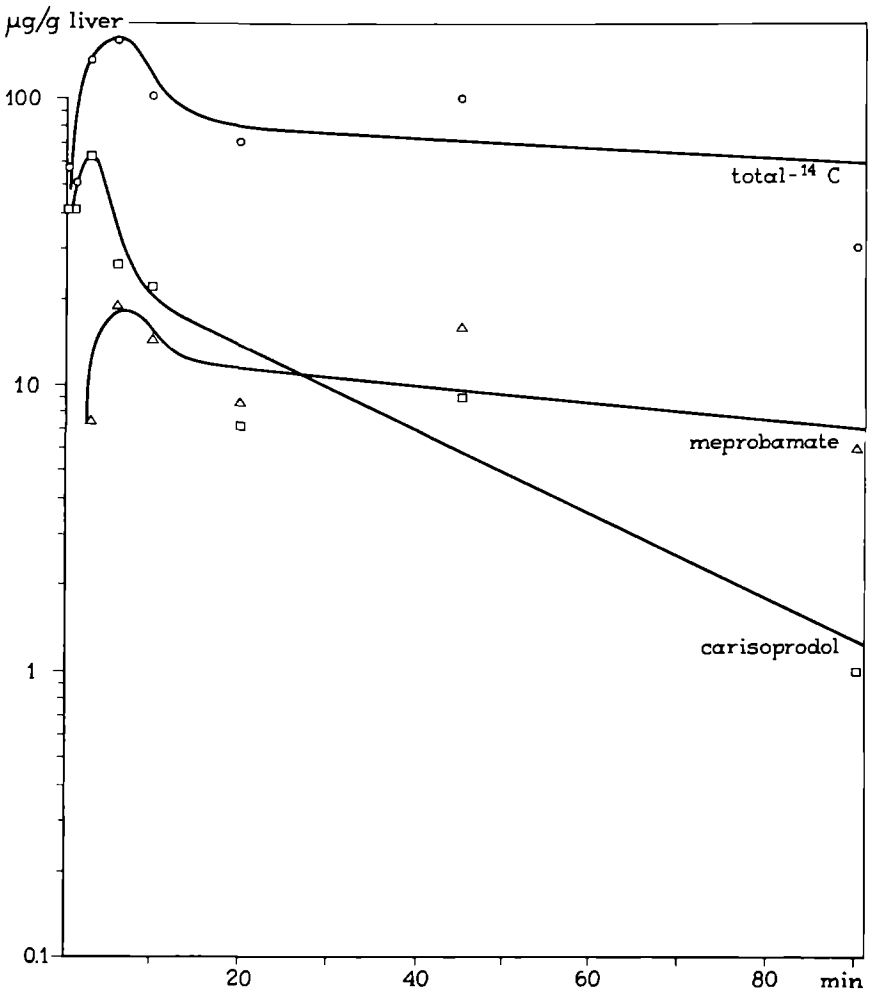

FIG. 13

Concentration decay curve of carisoprodol-^{14}C in liver of mice. In liver tissue next to meprobamate three other metabolites can be detected in minor concentrations.

agreement with the pattern established following the other routes of administration.

Metabolism of carisoprodol.

From tissue analysis it could be concluded that carisoprodol is rapidly

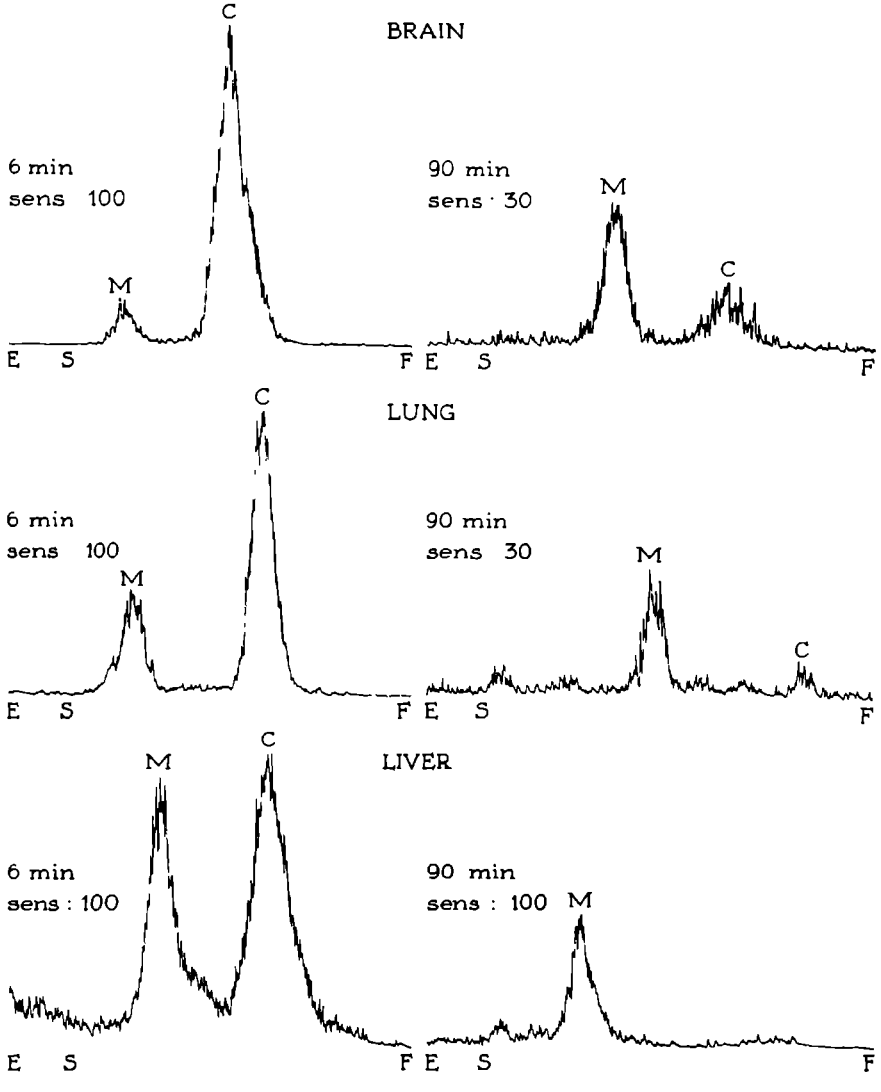

FIG. 14

Distribution pattern of carisoprodol and metabolites in tissue-extracts after separation on TLC-plates 10 min after i.v. administration. In brain, lung and heart the same pattern can be distinguished. In liver at least four metabolites can be observed.

dealkylated into meprobamate. Other metabolites were present in minor quantities. Only in liver other peaks corresponding to radioactive metabolites could be detected (FIG. 11, 12, 13).

Hydroxylation of carisoprodol and meprobamate was found to yield the major metabolites in dogs (EDELSON *et al.*, 1965).

Although the separated peaks were not closely investigated, the metabolites were quantitatively not of special interest in the brain, heart and lungs (FIG. 14). Maximum concentration of radioactivity in the tissue homogenates was reached 1-3 min after injection.

The concentration decay of carisoprodol in the tissue demonstrated a biphasic character. 10 min after administration the concentration of carisoprodol accounted for about 10 % of the maximum which is autoradiographically not detectable. The quantitative analysis data explain the great similarity of the autoradiograms of meprobamate and carisoprodol some 10 min after administration.

DISCUSSION

By qualitative comparison of the macroautoradiograms of mice which were given intravenously meprobamate, carisoprodol or tybamate, it appeared that lipid-solubility corresponding to partition coefficients and solubility in various solvents (DOUGLAS *et al.*, 1964; VAN DER KLEIJN, 1969) plays an important role in the rate of penetration of a drug into the brain tissue. Thus, uptake of meprobamate into the central nervous system was slow and slight, whereas carisoprodol and especially tybamate showed high central concentrations before 5 min after intravenous injection.

In the distribution in the brain at short survival periods vascularity plays an important role.

As a consequence inferior colliculi, cortex, thalamus, midbrain and hypothalamus showed the highest labeling (CASSANO *et al.*, 1967; GOLDSTEIN *et al.*, 1968).

However, differences in the regional distribution between meprobamate, carisoprodol and tybamate can only be explained on the basis of lipophilic properties and tissue affinity. The generally retarded uptake of lipophilic drugs in adipose tissue was slightly demonstrated during a short period from 5-10 min after injection. Rapid metabolism, however, controlled the elimination of the parent drug.

Dealkylation, hydroxylation and subsequent conjugation were reported as the main routes of biotransformation of drugs of the meprobamate-group (DOUGLAS *et al.*, 1962, 1963, 1966).

From tissue analysis of carisoprodol, quantitative differentiation of radioactivity demonstrated that after 10 min hardly any parent drug contributed to the observed autoradiographic pattern. Fig. 1, 2, 3 and 8 demonstrate the great resemblance of the patterns of radioactive material due to meprobamate and homologues after 10-30 min with the pattern of urea after 1 hour. This comparison leads to the conclusion that meprobamate and the metabolites of carisoprodol and likely tybamate are distributed over the total body water space.

Data of BERGER *et al.*, 1959 and of BERGER *et al.*, 1964 of paralysing activity and acute mortality, demonstrating a trend of increased toxicity and activity in the sequence of meprobamate, carisoprodol and tybamate after intravenous administration, can be explained on basis of penetration rate into the brain. The minor differences in toxicity and activity after oral administration agree with the autoradiographic and analytical data.

Biotransformation competes with the absorption process. The rate of metabolite appearance or metabolite formation is greater than the absorption rate.

Only when absorption rate is much higher than the rate of elimination enteral administration may demonstrate the observed differences in pharmacological properties.

Although these distribution studies give no correlation with the different shades in pharmacological action on the central nervous system it can be concluded that the faster penetration into the brain and fatty tissues in the body after i.v. administration can be explained by the increased lipophilic nature of the drugs.

The slow and slight penetration of meprobamate into the central nervous system lead to the comparison of the distribution patterns of the ataractic drugs with those of body-volume indicators.

Macroautoradiography, in general, does not enable the quantitative analysis of tissue concentrations.

From Fig. 8 it appears that there is a great similarity between the distribution of urea and meprobamate. Differences concern the time course in which the maximum brain concentration is reached.

Phenazon (antipyrine) penetrates more rapidly into the central nervous system. The rate of penetration of the compounds into the brain corresponds with the physicochemical properties expressed as solubility in water and in chloroform, as partition coefficients between chloroform and water and as R_f-value on T.L.C. (TABLE II).

High lipid-solubility, partition coefficient and R_f value parallels rapid penetration into the brain.

TABLE II

	Solubility 1 g/x g solvent		Partition Coefficient	R_f-value × 100
	chloroform	water	chloroform-water (1)	chloroform-aceton (q : 1)
UREA	0	1	0	2
MEPROBAMATE	200	300	3.3	19
PHENAZON	1	1 (3)	28 (2)	32
CARISOPRODOL	1.9	2050	400	55
TYBAMATE	0.5	4100	> 500	56

(1) Data of VAN DER KLEYN 1969 (2).
(2) Data of BRODIE (1964).
(3) Data of MARTINDALE, Extra Pharmacopoeia, R. G. Todd (ed.), The Pharmaceutical Press, London.

The urea and phenazon patterns are model for the distribution in total-body water although each of them show their own specific properties.

Body fluid compartments are classified as total body water 61 %, which is built up by intracellular water (34 %) and extracellular water (27 %). Of the extracellular water 15 % is the physiologically active part.

Of this 4 % is the plasma-water and 11 % the fast exchangeable interstitial water (MERZ, 1962).

The great volume variations between these intravasal, extracellular and total body water spaces enable a qualitative distinction of the distribution of drugs. Inulin (MW 5000-5500) is distributed over the physiologically active extracellular water and the distribution of dextran (MW 60.000-90.000) is restricted to the intravasal space, although little will leak to the extracellular space (FIG. 9). The distribution of inulin in e.g. skeletal muscle, myocardium and salivary gland is slight and in the pattern of dextran, radioactivity can predominantly be demonstrated in the blood-vessels.

CONCLUSIONS

Comparative investigations on the distribution of ataractics of the meprobamate-group in mice demonstrated that differences in lipophilic nature of the homologues resulted in variations in initial penetration rate of the drugs into the brain. Rapid metabolic conversion is considered as the reason that these variations cannot be demonstrated after enteral

administration of the compounds. The slow transfer through the placenta inhibited the uptake of the parent drug by the foetus.

Summary

The kinetics of distribution and metabolism of meprobamate, carisoprodol and tybamate were studied in mice following different routes of administration, according to the "whole body" autoradiographic technique of Ullberg (1954, 1958). The rapid penetration into the brain as observed for the homologues of meprobamate after intravenous administration at short survival periods can be correlated with the higher lipid solubility. A great resemblance of the pattern of the various compounds could already be observed 10 min after injection. Rapid biotransformation of carisoprodol in mice was established by analysis of tissue-extracts. Meprobamate was found to be the major metabolite in mice. Following rectal administration no mutual differences in distribution pattern could be noticed.

In pregnant mice a slow penetration of the drugs through the placenta was observed. Radioactivity was uniformly distributed in the foetus.

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CHAPTER V

KINETICS OF DISTRIBUTION AND METABOLISM OF DIAZEPAM AND CHLORDIAZEPOXIDE IN MICE

INTRODUCTION

Since the introduction of chlordiazepoxide (Librium®) in the treatment of anxiety and psychic tensions, 5-phenyl-1,4-benzodiazepines have been shown to be of great value in an increasingly wide area of therapeutic application.

Diazepam (Valium®) related to chlordiazepoxide in chemical structure and pharmacological actions is now preferred by many clinicians as the best anti-anxiety drug at present available and by its muscle relaxant properties.

Although there is an enormous amount of literature available concerning the clinical and experimental applications of the diazepines, only relatively few investigations on the distribution of diazepam, chlordiazepoxide and its metabolites in the body have been reported.

The metabolism of diazepam has been studied in animals and man. N_1-desmethylation, C_3-hydroxylation of the parent compound and conjugation of the hydroxylated products have been demonstrated as the major metabolic pathways in man, dog and mouse (DE SILVA *et al.*, 1964; RUELIUS *et al.*, 1965; SCHWARTZ *et al.*, 1965; DE SILVA *et al.*, 1966; KVETINA *et al.*, 1968). Para-hydroxylation of the 5-phenyl-ring was reported by JOMMI *et al.* (1964) for rabbit and proved by SCHWARTZ *et al.* (1967) for rat.

Differences in physico-chemical properties are used to explain the differences in pharmacokinetics of structure-related compounds (MAYER *et al.*, 1959; BRODIE *et al.* 1960; CASSANO *et al.*, 1967; HESS *et al.*, 1968; VAN DER KLEIJN, 1968).

The purpose of this investigation was to study the distribution of diazepam and chlordiazepoxide in mice at different survival periods, following different routes of administration.

Metabolism of diazepam was studied and differences in distribution

patterns were correlated to physico-chemical properties of the drugs and metabolites.

MATERIALS AND METHODS

Radioactive compounds:

^{14}C-diazepam and ^{14}C-chlordiazepoxide were generously supplied by Hoffmann-La Roche, Basle, Switzerland. The compounds were labeled in position 2 and had a specific activity of 12.3 μC/mg and 39.8 μC/mg respectively.

The chemical and radio-chemical purity of the compounds were checked by thin-layer chromatography in heptane-chloroform-ethanol (5:5:1) and by subsequent scanning with a Desaga-scanner type (12-2). Both labeled diazepam and chlordiazepoxide appeared to contain less than 2 pCt impurities.

Since ^{14}C-chlordiazepoxide is partially transformed by β-irradiation and daylight into the N_4-C_5-epoxide derivative, the compound was used immediately after delivery. Thin-layer chromatography is reported not to be useful to separate chlordiazepoxide from its epoxide derivative (KOFCHLIN *et al.*, 1965).

a) *Autoradiography.*

Adult, male Swiss mice (average weight 20-22 g) and pregnant female mice two days before delivery (bodyweight about 40 g) were used.

Of diazepam and chlordiazepoxide 15 mg (200 μC)/kg was given intravenously (i.v.) and 30 mg (200 μC)/kg was given orally.

The compounds were dissolved in dichlormethane containing polysorbate 80. The solvent was evaporated and the residue solubilized in 0.9 % NaCl-solution. The final polysorbate-concentration was 2 %. The mice received 0.01 ml/g bodyweight. Immediately before sacrificing the mice were lightly anaesthetised with halothane and rapidly frozen by immersion in isopentane cooled to about -70°C with solid carbon dioxide

After i.v. injection survival periods of 14″, 50″, 60″, 2′, 5′, 10′, 15′, 30′, 45′, 60′, 2 h, 4 h, 6 h, 7 h, 16 h, 24 h were used in male mice, while in pregnant mice survival periods of 1′, 3′, 5′, 10′, 15′, 30′, 60′, 2 h, 4 h and 15 h were used; after oral administration survival times of 5′, 15′, 30′, 60′, 90′, 2 h, 3 h, 4 h, 7 h and 24 h were used

Autoradiographic technique according to Ullberg (1954, 1958) was used. The frozen animals were embedded in cooled carboxymethylcellulose (5 %) solution, and rapidly frozen in solid carbon dioxide. Sagittal sections, 30 μ thick simultaneously through two animals were made in a refrigerated room (temperature about -12°C) with an hydraulic driven Leitz-sledge microtome 1300. After adhering to Scotch Tape (Permanent Mending Tape No. 810, 3 M Co., U.S A.) the sections were freeze-dried and pressed on to Structurix (Gevaert) D 7 X-ray films and exposed for 15-25 days at -12 °C

b) *Chromatography.*

Thin-layer chromatography on precoated TLC-plates, Silica Gel F 254 (Merck A G., Darmstadt, Germany) was done while the following solvent systems were used :

1. Heptane-chloroform-ethanol (5:5:1).
2. Benzene-ethanol (96 %) (8.5.1.5).
3. Chloroform-ethanol (abs.) (9:1).

In order to improve separation TLC-plates were developed three times successively.

Tissue analysis.

Male Swiss mice, previously starved for 24 hours, were injected intravenously in the tail

vein with diazepam (27 2 mg (340 μC/kg), and killed 1′, 5′, 10′, 15 , 30′, 45′, 60′, 2 and 4 h after administration The brains, hearts and livers were removed and weighed and subsequently homogenized with an Ultra-Turrax TP 18/2, in 2 vol of a 0 5 M phosphate buffer (pH = 6 8), containing 0 1 % KCN The tubes and homogenizer were rinsed The decanted homogenates and rinsing fluids of each sample were collected and made up to 10 0 ml 1 00 ml of homogenate was transferred into a countvial and assayed for total radioactivity The remaining portion of the tissue homogenates was transferred into 40 ml tubes and extracted twice with 10 ml of ethylacetate The organic phases were collected and evaporated The residues were transferred quantitatively to thin-layer chromatography plates and developed once in solvent 2 and subsequently twice in solvent 3

The efficiency of the extraction accounted for 60 % of the total radioactivity

Urine analysis

Urine pooled from animals used in the autoradiographic experiments was extracted with ethylacetate and the ethylacetate-residues were transferred to thin-layer chromatography plates for identification These plates were chromatographed three times successively in solvent 3

Assay of radioactivity

The radioactivity of the homogenates was counted after mixing with 1 0 ml of 0 75 M Soluene® (Packard) and warming for 45 min at 50°C After subsequent mixing with 15 0 ml of scintillation cocktail of Bray (1960) the counting was performed with a Nuclear Chicago, Mark I, liquid scintillation counter Corrections were made by the external standard method

Radioactive bands on TLC-plates were detected with a Desaga scanner (type 12-2) equipped with a Berthold Zahlratenmesser LB 2031 and synchronously recorded

Quantitation of the scans was performed by estimating peak areas as the product of peak height, and width at half height

Spots and bands of intact diazepam were removed from the plates and suspended in 10 0 ml of a scintillation cocktail containing 4 g PPO, 0 250 g dimethyl-POPOP, 30 g CAB-O-SIL in 1 L toluene and assayed

Peak-areas of the separated diazepam and derivates were correlated with the data obtained from liquid-scintillation-counting of the diazepam-spots

The efficiency of the TLC-procedure accounted for 95 6 % The efficiency of the total procedure of extraction, spotting, developing, scraping and scanning was 57 %

Results

Whole-body autoradiography following intravenous and oral administration

The uptake of radioactive diazepam in the brain was already very high, 14 sec. after i.v. injection, while the uptake of chlordiazepoxide was slightly lower. Survival periods shorter than 14 sec. could not be studied. At the early survival periods extremely high concentrations of radioactivity were found in myocardium, cervical muscle, adrenal and kidney

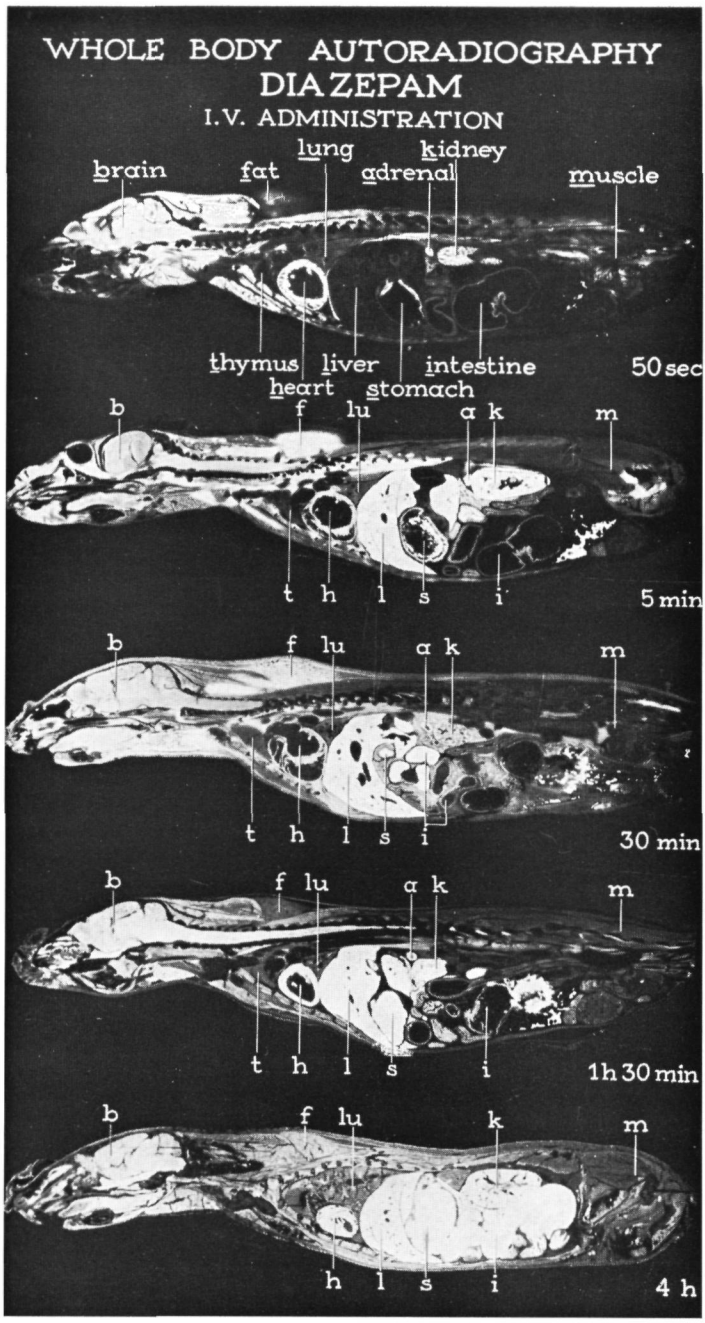

FIG. 1

Autoradiograms showing the distribution of ^{14}C-diazepam and radioactive metabolites in mice after i.v. injection of 15 mg (200 μC)/kg.

White areas in the autoradiograms correspond to high activity. A very rapid uptake in CNS, kidney, adrenal, stomach wall, cervical muscle and myocardium can be observed.

A low concentration is detectable in the blood. The liver and the bodyfat show an increasing concentration of radioactivity during the first 5 min. period. The gastro-intestinal tract shows an increasing amount of radioactivity at all survival periods after 5 min. after injection.

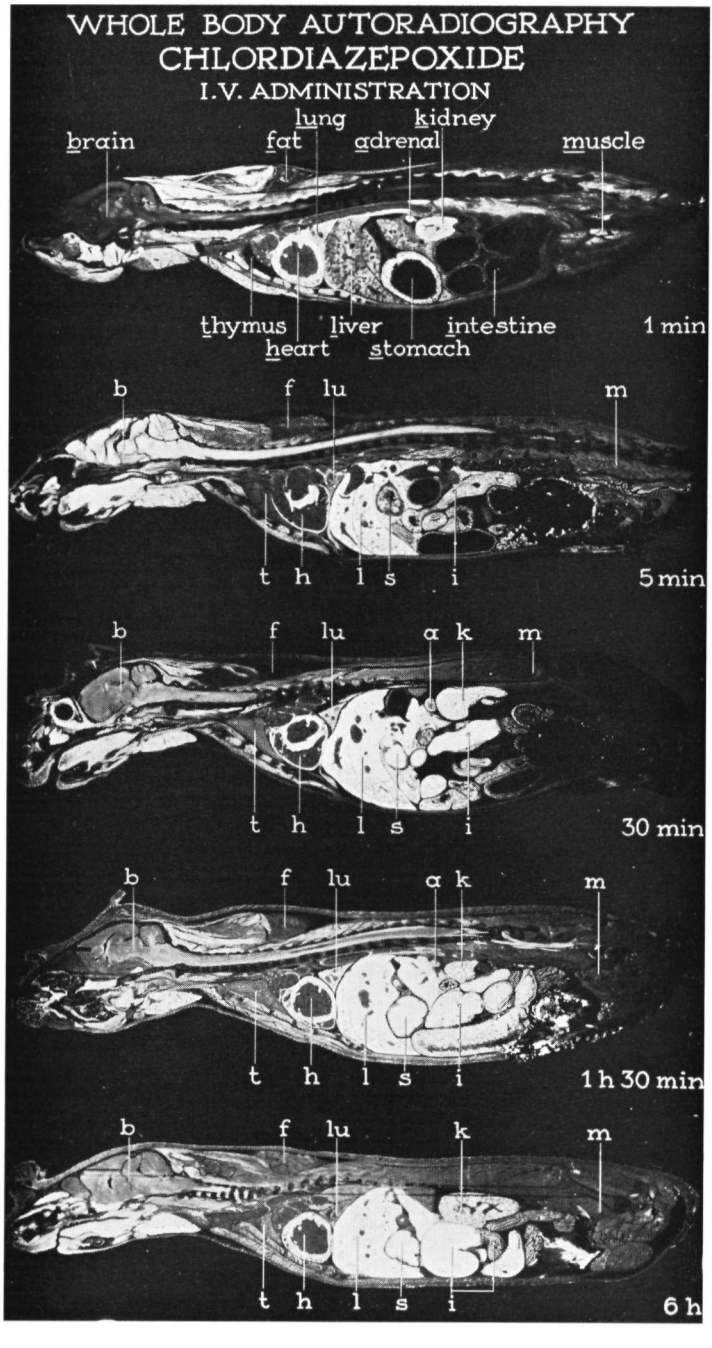

FIG. 2

Autoradiograms showing the distribution of ^{14}C-chlordiazepoxide and radioactive metabolites in mice after i.v. injection of 15 mg (200 μC)/kg.

White areas in the autoradiograms correspond to high activity. The radioactive material is rapidly taken up by the adrenal, kidney, cervical muscle, stomach wall and myocardium. The liver and the brain show an increasing concentration of radioactivity during the first 5 min. period. The gastro-intestinal tract shows an increasing amount of radioactivity at all survival periods after 5 min. after injection.

after administration of diazepam or chlordiazepoxide. The liver showed a spotted pattern, till 2 min. after i.v. injection. In the first periods after administration vascularity and affinity for the drug play an important role in the uptake of the radioactive labeled drugs by the different organs and tissues (FIGS. 1 and 2).

From 3-30 min. after i.v. administration a high and constant level of radioactivity was present in the liver, kidney, salivary and lachrymal

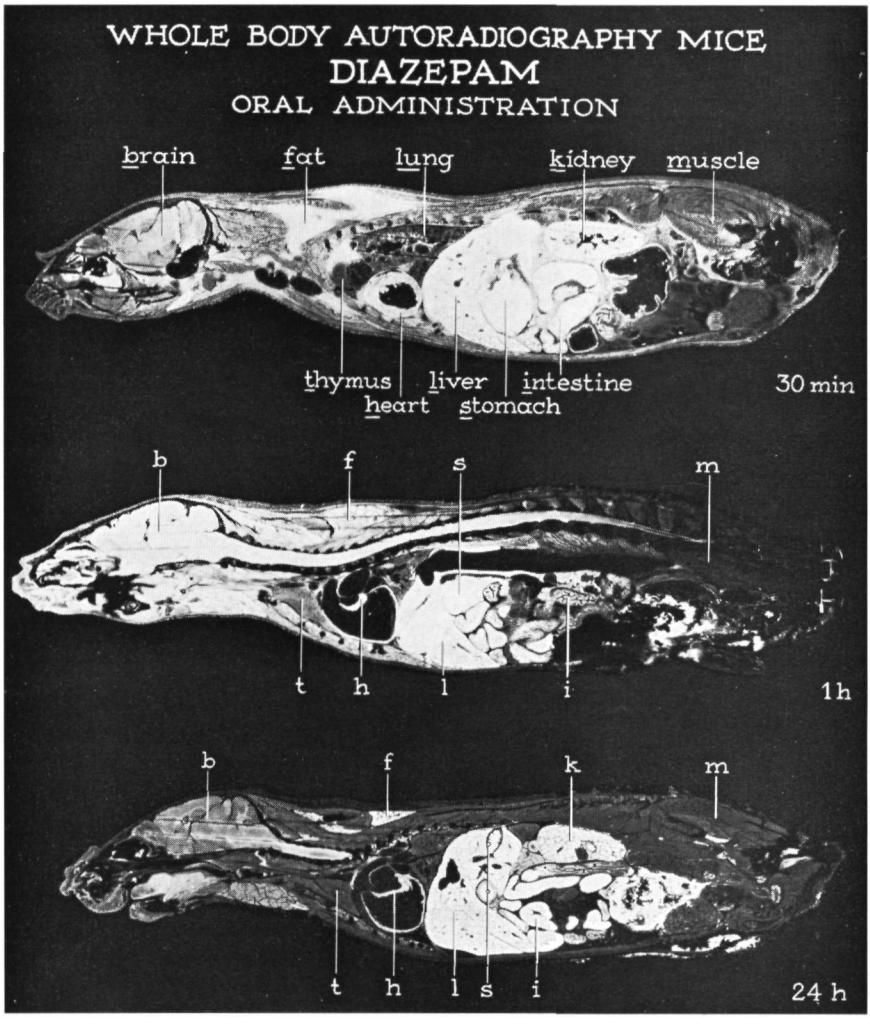

FIG. 3

Autoradiograms showing the distribution of ^{14}C-diazepam after oral administration of 30 mg (200 μC)/kg. Note the high uptake of diazepam in the brain, heart, adipose tissue and liver at 30 min. after administration.

glands, heart and skeletal muscle, great vesselwalls and adrenal cortex. The initial labeling of different regions in the brain was leveled out. A different distribution pattern in the brain occurred after 30. min.

From 30 min.-4 h after administration there was a slow decrease in the concentration of radioactivity in all organs. The concentration decay of radioactivity was faster in the case of chlordiazepoxide than of diazepam. The ratio between the concentrations of radioactivity in brain and blood was higher after i.v. and oral administration of diazepam than after similar administration of chlordiazepoxide at each survival period respectively as could be measured densitometrically.

The concentration of radioactivity derived from diazepam fell only slightly during the first 4 h period in all organs and rose in the gastrointestinal tract, while after 24 hours only small amounts of the drugs were detectable in the excretory organs, the peripheral nerves and the nasal cavity.

The general pattern of distribution at 2 h following oral administration of diazepam or chlordiazepoxide was similar to that seen after i.v. injection (FIG. 3). At shorter survival periods obviously phase differences due to the absorption process could be detected. 5 Min. after oral administration the brain and especially the gray matter demonstrated its high affinity for both drugs. This affinity was more pronounced in the case of diazepam than in the case of chlordiazepoxide.

Accumulation of the drugs in the brain, liver, muscles, heart and kidney was progressive and reached a maximum some 1-2 hours after oral administration.

Retention of labeled material was observed likewise in the white matter of the central nervous system after 4 hours.

15 Min. after ingestion labeled diazepam appeared in the adipose tissue. The maximum concentration was reached in 1 hour and decreased rapidly there after.

Specific aspects of distribution of diazepam and chlordiazepoxide

Central nervous system :

Since diazepines are considered to affect specific functions of the central nervous system, a detailed investigation of the distribution of the drugs throughout the brain was desirable.

After i.v. administration there is a rapid uptake of radioactivity by the gray matter of the cerebral and the cerebellar cortex, especially the

colliculi and the thalamic and medullar nuclei. The uptake of diazepam into the brain was somewhat faster than that of chlordiazepoxide.

The concentration of ^{14}C-diazepam reached its maximum in about 1 min. after i.v. injection., whereas the uptake of ^{14}C-chlordiazepoxide increased progressively for 5 min. after administration (FIG. 4 and 5).

From 5-30 min. the predominant labeling of the gray matter by both compounds has become equivalent over the whole brain, whereas after 30 min., selective retention of radioactivity started in the white matter

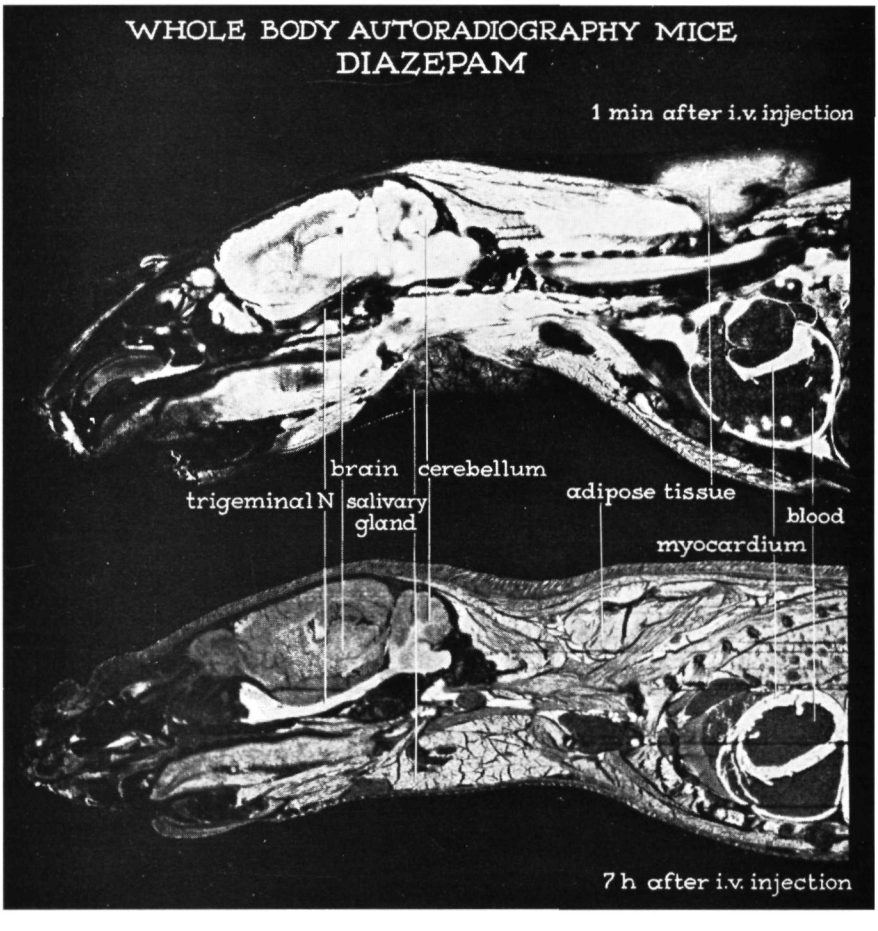

FIG. 4

Details of autoradiograms showing the distribution of ^{14}C-diazepam and radioactive metabolites in mice. Note the high uptake of radioactivity 1 min. after i.v. injection in the cortex of cerebrum and cerebellum in the colliculi and in the thalamic and medullar nuclei. The labeling of the adipose tissue is typical for diazepam. After 7 hours the concentration in the brain is equaled. Retention in the white matter and peripheral nerves can be observed.

especially that of the corpus callosum, midbrain, cerebellum and spinal cord. Radioactivity was detectable in the peripheral nerves : the trigeminal, optic and spinal nerves, for up to 24 h after administration.

The distribution patterns of radioactivity throughout the anatomically corresponding regions of the brain were similar for both compounds, although loss of radioactivity derived from chlordiazepoxide was faster than that from diazepam.

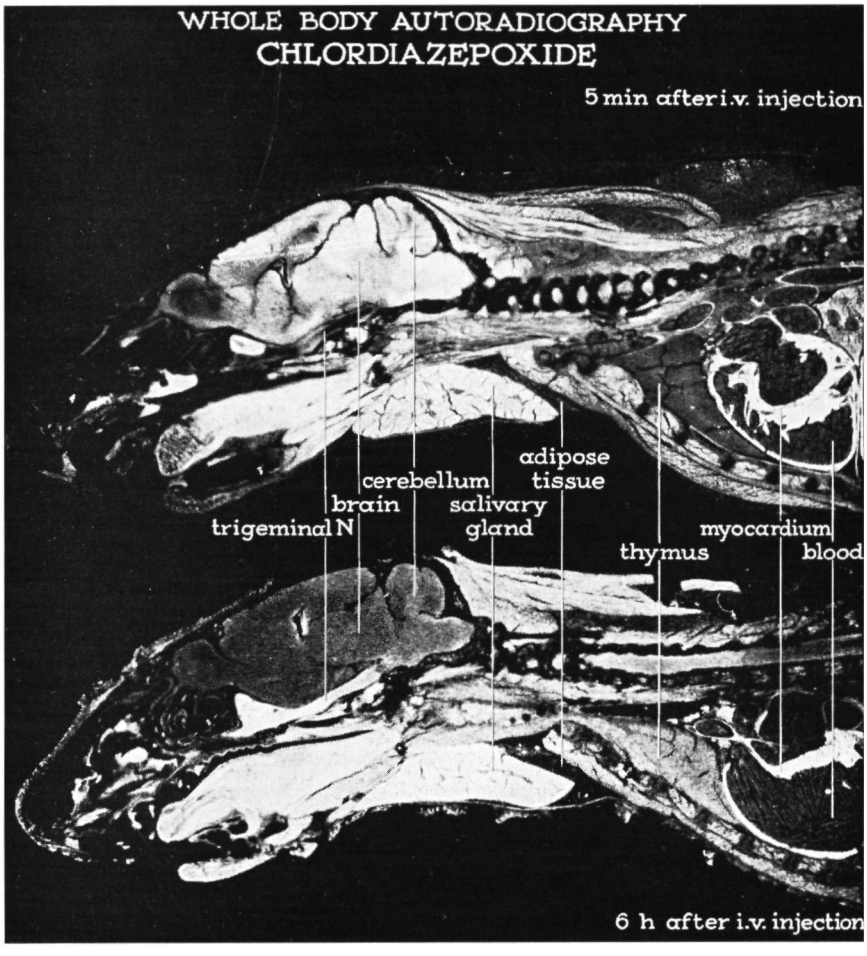

FIG. 5

Details of autoradiograms showing the distribution of ^{14}C-chlordiazepoxide and radioactive metabolites in mice. Note the selective uptake of radioactivity 5 min. after i.v. administration in the colliculi and the thalamic and medullar nuclei of the brain. No labeling of the adipose tissue can be observed. After 6 hours the radioactivity is retained in the peripheral nerves e.g. the trigeminal and optic nerve.

Cardiovascular system.

The labeled compounds were taken up very rapidly by the myocardium. Indeed, the heart muscle and great vessel walls showed a very high concentration of radioactivity within 14 sec. after i.v. injection. The high concentration of radioactivity remained over the six hour period after which a gradual decrease was demonstrated.

In the spleen no differences were observed between the labeling of the white and red pulp. The concentration of radioactivity in the spleen was somewhat higher than in the blood at all survival periods.

Liver.

The liver showed a spotted pattern characteristic of other lipophilic compounds at short periods after i.v. injection (CASSANO *et al.*, 1967; CASSANO and HANSSON, 1966).

During the first 5 min. a progressive increase of labeling was observed. After 5 min. the labeling remained extremely high and a slow decrease could be demonstrated after 6 hours.

Digestive system.

1 Min. after i.v. injection the mucosal wall of the stomach showed a high amount of radioactivity. 9 Min. later the contents of the stomach were strongly labeled indicating an abundant excretion of radioactivity into the lumen. Although the mucosa of the proximal as well as the distal part of the small intestine and coecum, colon and rectum were labeled more strongly than the serosa no excretion into the lumen of the gut was demonstrated.

From 30 min. after injection the lumen of the intestine contained increasing amounts of radioactivity. Most probably this radioactivity originates from the excretes of stomach and bile.

The experiments with oral administration of diazepam and chlordiazepoxide to mice demonstrated that the drugs only slowly absorb from the intestine. It is probable that this phenomenon also occurs in man and dog since it has been demonstrated that maximum blood concentrations are not obtained until 1-2 hours after oral administration (DE SILVA *et al.*, 1966, GARATTINI, 1968; VAN DER KLEIJN, 1969).

The absorption as well as the reabsorption of the drug will be discussed in the latter part of this paper.

Eye.

The eye rapidly accumulated the labeled material. Radioactivity due

to both drugs was detectable for several hours especially in the choroidal layer.

The labeling of the trigeminal and optic nerve has been already reported under CNS. This pattern of labeling is not specific to the diazepines since other strongly lipophilic drugs show the same phenomenon (CASSANO and HANSSON, 1966).

A high amount of radioactivity was present in the lachrymal glands and it is possible that lachrymal secretion contributes to the labeling of the nasal cavity and subsequently of the stomach.

Endocrine glands.

The pituitary built up a high level of radioactivity which commenced 1 min. after i.v. injection, and lasted no longer than 15 min.

The adrenal cortex contained more labeled drug than the medulla at all survival periods.

Urogenital system.

The radioactive drugs were rapidly taken up by the ovaries. The corpora lutea showed a high amount of labeled material during 6 hours after i.v. administration.

The uptake of radioactivity by the sexual organs of the male was not of special interest. A rather low and uniform distribution was observed. However, in the testicle a net structure, not specific for the diazepines could be observed.

The kidney showed a high labeling already during the early survival periods. The concentration of radioactivity decreased slowly over the next hours. After 5 min. radioactivity could already be observed in the urine bladder.

Lungs.

The radioactivity in the lungs was somewhat higher than that present in the blood, but comparable to that found in skeletal muscle, spleen and pancreas.

Lymphatic tissue.

In the lymph nodes and thymus a slow and slight accumulation of radioactivity could be demonstrated.

Skin.

Both compounds were taken up by the skin at a moderate rate. From 5 min. after i.v. administration high concentrations were noted in the hair folliculi.

Adipose tissue.

In contrast to chlordiazepoxide, diazepam was highly taken up in the brown fat as well as the perirenal and the epididymal fat. This is in agreement with recent findings of MARCUCCI *et al.* (1968 b). Although little radioactivity was present before 2 min., after i.v. injection, the uptake proceeded progressively for 30 min. After that period, the radioactivity fell more rapidly from adipose tissue than from the other organs. This indicates the removal of the original drug rather than the metabolites. After oral administration labeling of the body fat appeared within 15 min., but had almost disappeared after 2 hours.

Skeletal muscle.

The labeling of the muscle was built up slowly from the moment of injection till 10 min. later. This may be due to the relatively poor blood supply of muscle at rest and the rather low affinity of the tissue for the drugs. In contrast the cervical muscle already showed a high labeling 1 min. after i.v. injection. After 10 min. a rather constant level of radioactivity was observed in the muscles, which remained higher than that observed in blood.

Distribution of diazepam in pregnant mice

The passage of the drug through the placenta was slow and slight. After 15 min. a low amount of radioactivity appeared in the foetus. The maximum concentration was reached 2 hours after the i.v. administration.

The distribution of the labeled material in the foetus was rather uniform. The concentrations in corresponding tissues were lower in the foetus than in the mother at all survival periods. The liver of the foetus did not take up a high amount of radioactivity but the brown fat and the mucosa of the gastrointestinal tract contained a surprisingly high amount some 30 min. after injection (FIG. 6).

A considerable amount of radioactivity was still present in the foetus after 4 hours. However, the concentration remained lower than that present in the mother. After 15 hours radioactivity had almost disappeared from the foetus. At that time radioactivity could still be observed in the mother.

Metabolism of diazepam

From tissue analysis it could be concluded that diazepam is rapidly metabolized into several compounds detectable by their ^{14}C-label (FIGS.

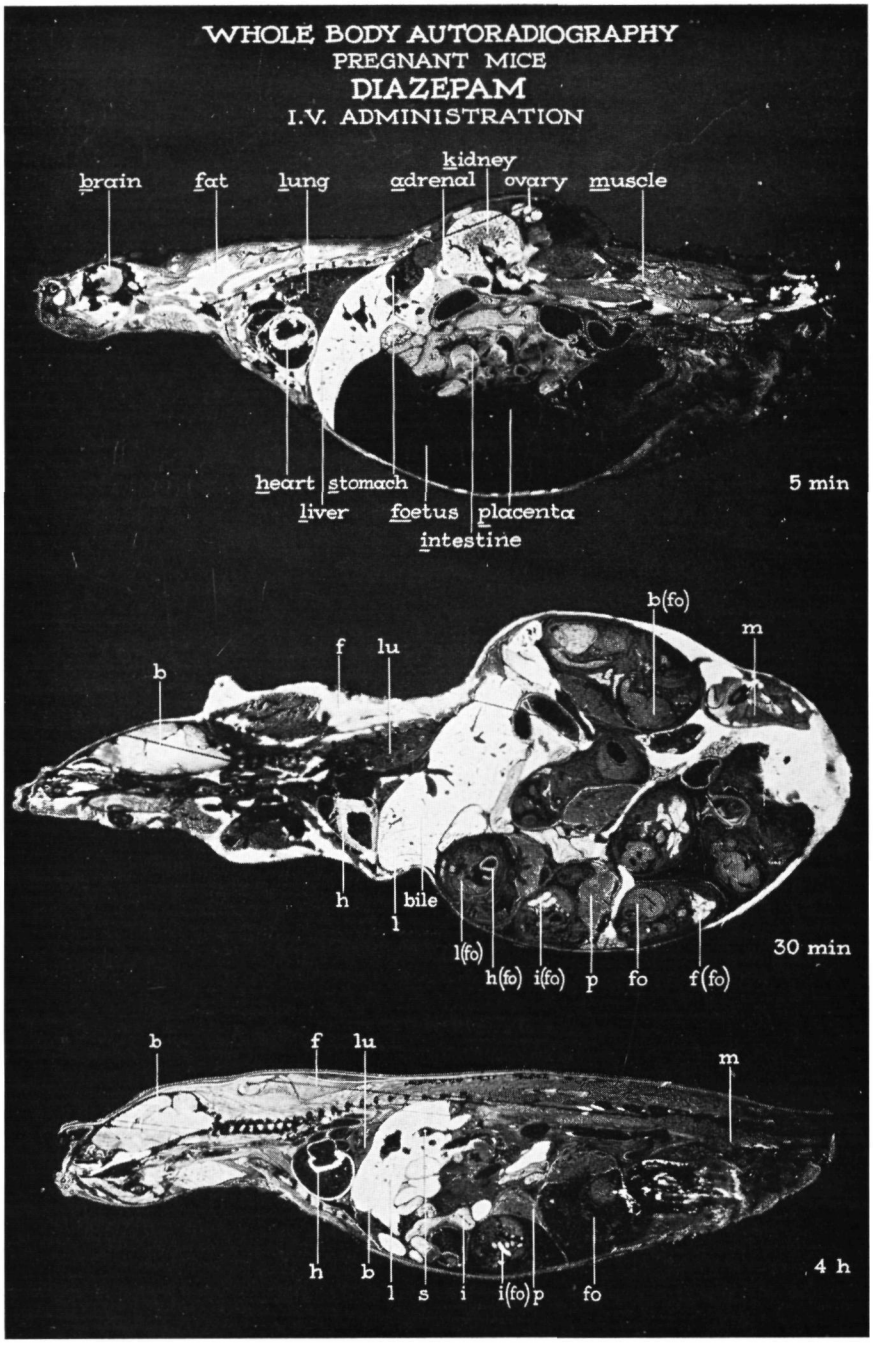

FIG. 6

Autoradiograms showing the distribution of ^{14}C-diazepam and radioactive metabolites at various survival periods in pregnant mice. Note the slow and slight uptake of radioactivity into the foetus. At later survival periods the highest amounts of radioactivity in the foetus are found in liver, bodyfat and gastrointestinal tract.

7, 8 and 9). Diazepam (1), oxydiazepam (2), desmethyldiazepam (3) and oxazepam (5) could be identified by the R_f-values of their reference compounds. Other metabolites were not identified structurally, but were reported by SCHWARTZ *et al.* (1967). Desmethyldiazepam was found to be the major metabolite in heart and brain, while oxydiazepam and oxaze-

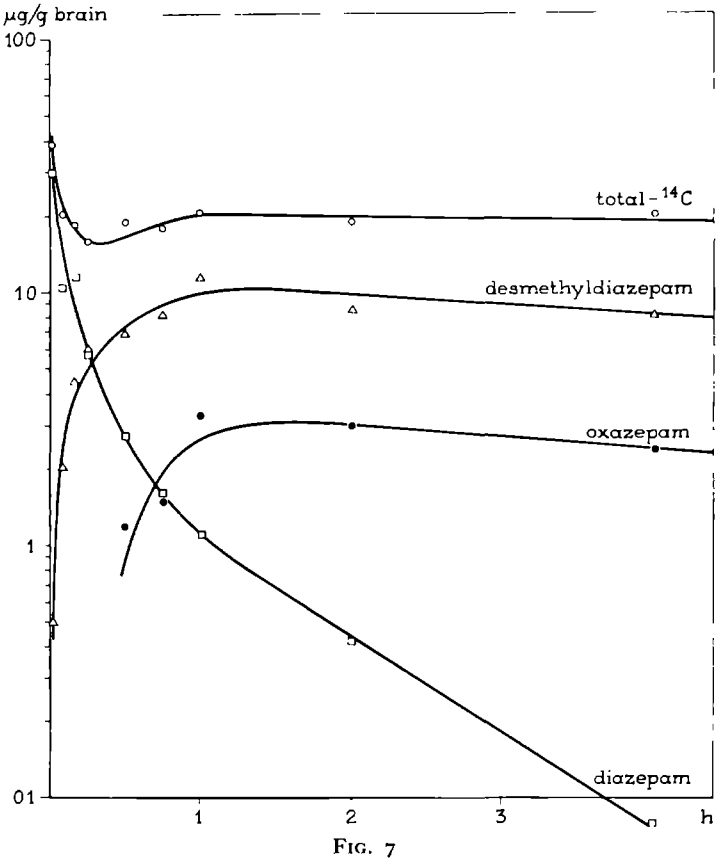

FIG. 7

Kinetics of metabolism and elimination of diazepam in brains of mice.
Concentration-time curve of total radioactivity expressed as μg diazepam/g tissue (○——○) and of diazepam (□——□) and its main metabolites, N$_1$-desmethyldiazepam (△——△) and oxazepam (●——●). Other metabolites are present only in minor quantities (see FIG. 11).

pam were found only in minor quantities. It may be concluded that desmethyldiazepam and the other metabolites are more slowly eliminated than the parent drug. However, in rat tissue desmethyldiazepam is detectable only in minor quantities (GARATTINI, 1968).

This indicates that different species have different rates of formation and elimination of metabolites. In man, desmethyldiazepam and

oxazepam appear only slowly in plasma while the rate of elimination of desmethyldiazepam is about equal to that of diazepam (DE SILVA *et al.*, 1966; GARATTINI, 1968; VAN DER KLEIJN, 1969).

In urine, hardly any original drug, minor quantities of desmethyldiazepam and oxydiazepam and large amounts of oxazepam and an

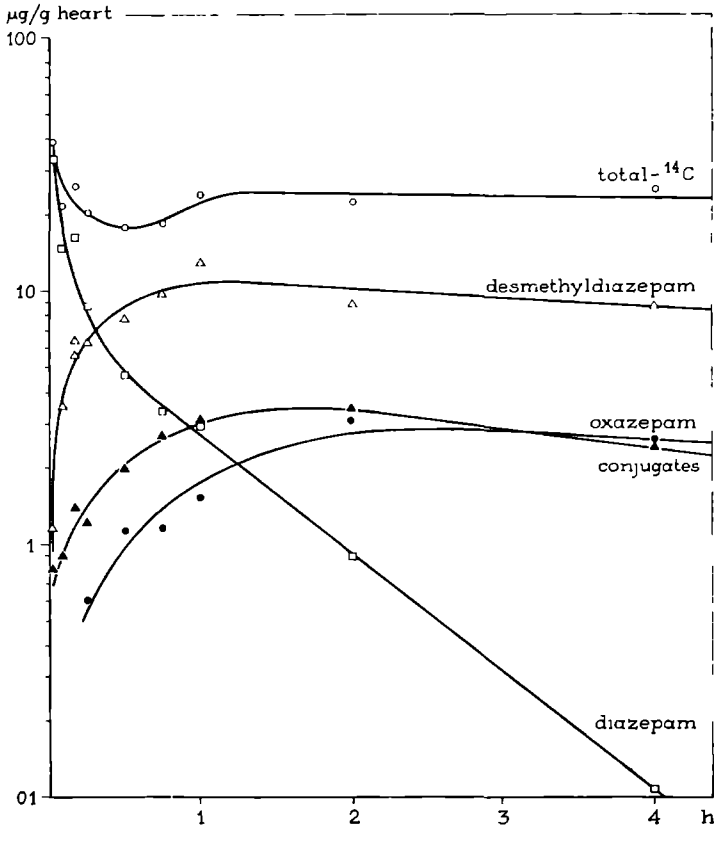

FIG. 8

Kinetics of metabolism and elimination of diazepam in hearts of mice.
Concentration-time curve of total radioactivity expressed as μg diazepam/g tissue (○——○) and of diazepam (●——●) and its main metabolites, N_1-desmethyldiazepam (△——△), oxazepam (●——●) and one or more unknown metabolites of high polarity (▲——▲) analysed in ethylacetate extracts of tissue homogenates. Other metabolites are present in minor quantities.

unknown metabolite (6) were detected. Closer examination of the area on the chromatogram between peak 4 and 5 and between 6 and 8 indicated the presence of four other unknown metabolites. In urine eleven radioactive metabolites could be separated (FIG. 10).

From figures 7 and 8 it appears that the elimination of diazepam has a

biphasic nature. The rate of elimination can be characterized by the half-life time ($t\frac{1}{2}$) or by the time constant τ [$\tau = 1.44 \times t\frac{1}{2}$]. The rate of appearance of the metabolites in the tissue can also be characterized by τ and is assumed to be correlated with the rate of metabolism.

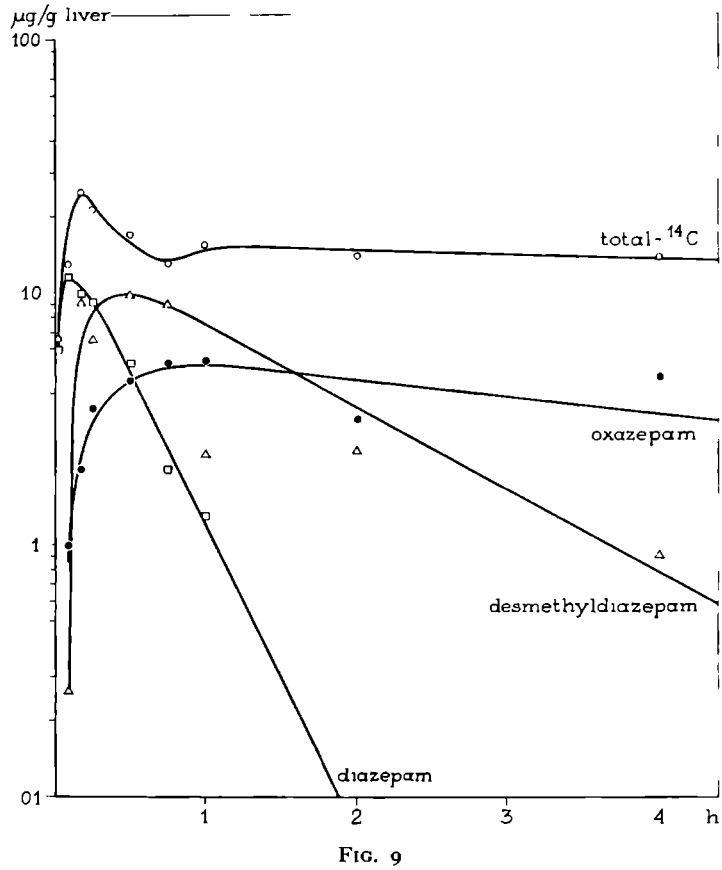

FIG. 9

Kinetics of metabolism and elimination of diazepam in liver of mice.

Concentration-time curve of total radioactivity expressed as μg diazepam g tissue (○——○) and of diazepam (·——□) and its main metabolites, N_1-desmethyldiazepam (△——△) and oxazepam (●——●) Other metabolites are present only in minor quantities. The relative amounts of diazepam and its metabolites present in the liver differ from the pattern present in heart and brain

Oxazepam reaches higher concentrations than in figures 7 and 8. From the pictures 7, 8 and 9 it can be concluded that in mice N_1-desmethylation of diazepam is a much faster process than C_2-hydroxylation.

To estimate $t\frac{1}{2}$ of the initial parts of the elimination curves of diazepam and the appearance curves of the metabolites use is made of the residual plot method (SOLOMON, 1960). From Table I it appears that the initial elimination of diazepam is very rapid. In this phase distribution of the

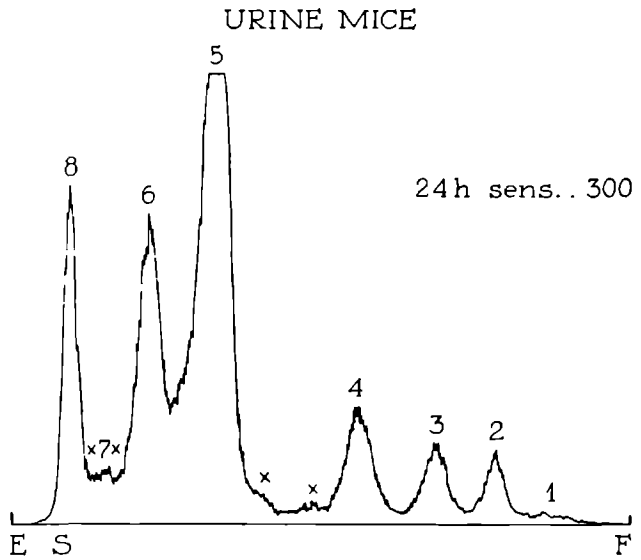

FIG. 10

Radioscan of an ethylacetate extract of pooled urine of mice treated with ^{14}C-diazepam. A trace amount of the parent compound is recovered. Oxazepam (5) and an unknown metabolite (6) are found in relatively high concentrations.

Seven metabolites are detectable in the extracts. TLC was carried out successively in solvent 2.2 and 2.

Closer analysis of the area between 4 and 5, and between 6 and 8 yielded four other metabolites (×).

TABLE I

	Diazepam		Desmethyldiazepam		Oxazepam	
$\tau = 1.44 \times t\frac{1}{2}$ min.	τ_1	τ_2	τ_{1m}	τ_2	τ_{1m}	τ_2
Brain	10	68	17	> 240	76	> 240
Heart	7	57	23	> 240	26	> 240
Liver		21	12	± 80	16	> 240

Table summarizing some kinetic parameters of metabolism and elimination of diazepam in mice calculated from the curves in the figures 7, 8 and 9 according to the residual plot method (Solomon, 1960). The time constants of appearance of the metabolites in the tissue are supposed to be equal to the time constant of metabolite formation. The elimination of diazepam occurs in two phases. The first phase parallels the formation of the metabolites. The second phase is supposed to be highly controlled by the reabsorption process. τ_1 = time constant of elimination process, first phase. τ_2 = time constant of elimination process, second phase. τ_{1m} = time constant of metabolite formation process.

drug over a large apparent distribution volume takes place and rapid metabolism greatly controls the elimination of the parent drug. The elimination rate of the two most important metabolites in mice is much lower than that of diazepam.

In the liver concentrations of the metabolites are different from those in the heart and brain. This might indicate that the liver is the main locus of metabolism.

The reduced lipophilic nature of the desmethylated and hydroxylated compounds makes the presence of these compounds in the brain not impossible (FIG. 11). It appears that the pattern of radioactivity can only partly be due to the radiation of the original compound. After 1 hour only about 10 % of the radioactivity is present as diazepam.

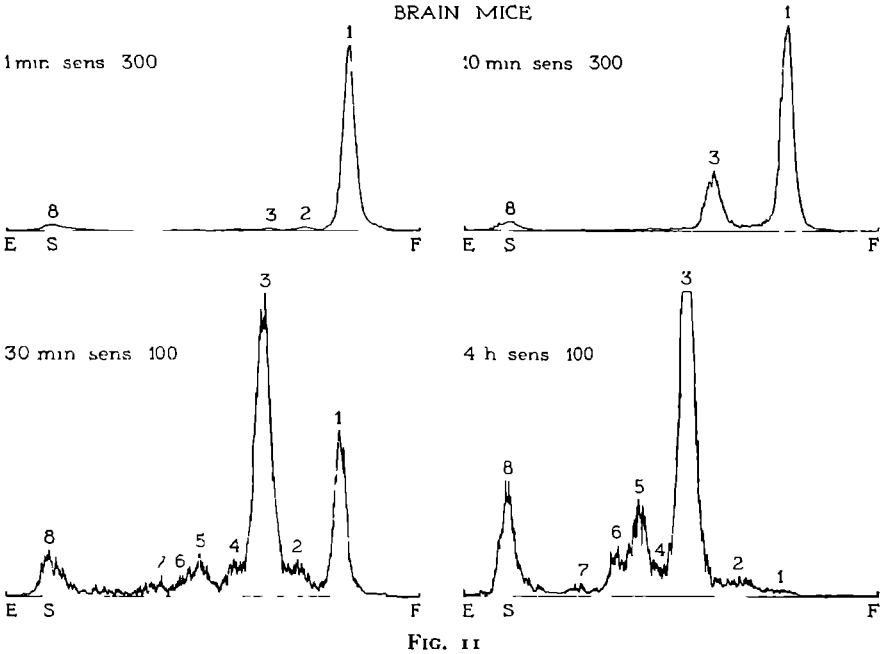

FIG. 11

Some characteristic radioscans of brain extracts demonstrating the ethylacetate-extractable radioactivity. At least seven metabolites can be detected.

Diazepam (1), oxydiazepam (2), N_1-desmethyldiazepam (3) and oxazepam (5) are identified by R_f-values of the reference compounds.

Metabolism of diazepam causes the formation of compounds with lower R_f-values than the parent compound. TLC was carried out successively in solvent 2, 3 and 3.

E = edge of plate; S = start; F = solvent front.

DISCUSSION

Comparison of the autoradiograms after i.v. and oral administration at successive survival periods is useful although tissue analysis has made

clear that C_2-^{14}C-diazepam and C_2-^{14}C-chlordiazepoxide are rapidly converted into compounds still containing radioactive label. 3-Hydroxylation an N_1-desmethylation is reported not to result in significant pharmacological inactivation but in decreased activity and toxicity (MARCUCCI *et al.*, 1968 a). So the pictures continue to be representative for the distribution of the ataractic substance.

Although no great differences in pharmacological effect between diazepam and chlordiazepoxide could be observed visually in mice, it appeared from our autoradiograms that in the case of diazepam a faster penetration into the brain than of chlordiazepoxide could be demonstrated. This observation is plausible since diazepam has a stronger lipophilic nature than chlordiazepoxide (VAN DER KLEIJN, 1969).

Recently PLACIDI and CASSANO (1968) published whole-body autoradiographic studies on chlordiazepoxide. From their pictures a more rapid accumulation of the drug in the brain could be demonstrated. Differences in dose of radioactive material may be the cause of this discrepancy.

The distribution in the brain is evidently of special interest. The high concentration of diazepam and chlordiazepoxide in brain cortex, thalamus and brainstem of the mice may explain the initial narcotic and paralysing effect which last for about 3-5 min. After recovery no special inhibited behaviour of the mice could be observed visually. After 45 min. and more pronounced at later survival periods more detailed differences in the brain pattern of radioactivity were found.

The white matter, especially of the spinal cord, of cerebellum and cerebrum, in the corpus callosum, brainstem and the peripheral nerves, e.g. the trigeminal and optic nerves, was stronger labeled than the surrounding tissue. It is unlikely that unchanged diazepam will contribute to this detailed labeling to a considerable extent since one hour after administration about 90 % of the original drug is converted into at least seven metabolites of which desmethyldiazepam is the most important one (FIG. 7).

The weak basic and lipophilic properties of chlordiazepoxide, diazepam and their metabolites may explain the abundant excretion of the drugs into the strongly acid lumen of the stomach in agreement to the pH-partition theory (BRODIE, 1964).

The enteric circulation of the drug with contribution of excretions from the nasal cavity, stomach, bile and possibly intestinal glands caused a rapid inflow of radioactivity into the gastro-intestinal tract. The strong binding nature of the compounds to proteins and other macromolecules may indicate that the drugs can be bound to elements of the intestine.

The presence of radioactivity in the mucosa of stomach and intestine of the foetus starting about 30 min. after administration of the drug may confirm this.

These phenomena may cause a slow and sustained absorption of the drug from the intestine. Pharmacokinetic data of chlordiazepoxide and diazepam in man and dog indicate a reaching of the maximal blood-concentration not before 1-2 hours after administration (KOECHLIN and d'ARCONTE, 1963; DE SILVA *et al.*, 1964; KOECHLIN *et al.*, 1965; SCHWARTZ *et al.*, 1965; DE SILVA *et al.*, 1966; GARATTINI, 1968; VAN DER KLEIJN, 1969). Since metabolic transformation only gradually affects the psychopharmacological properties the absorption process may contribute to a prolonged time course of action of diazepam and chlordiazepoxide.

The difference in distribution in adipose tissue between diazepam and chlordiazepoxide can also be explained by the differences in lipophilic nature. The high accumulation of radioactivity in the brown and white fat of mice and of foetus after i.v. injection as well as after oral administration of diazepam at relatively short survival periods and the rapid removal at later periods do suggest that this accumulation predominantly is caused by intact diazepam more than by its metabolites.

The spotted pattern of the liver at the early survival periods after i.v. injection may be explained by the fact that only the arterial circulation contributes to the pattern and that the portal circulation causes a phase difference.

The high affinity of the liver for diazepam may be involved in the effect of the drug on the bromosulphophthalein liver test *in vitro* (KVETINA *et al.*, 1968).

The accumulation and long retention of radioactivity in the liver might suggest an induction of drug-metabolizing enzymes on the liver microsomes by diazepam that have been reported for chlordiazepoxide (HOOGLAND *et al.*, 1966).

Although from electrophysiological experiments conclusions have been drawn concerning the site of central depressant action (PRZYBYLA and WANG, 1968), the distribution pattern in the brain after different survival periods is insufficient to explain these data.

SUMMARY

The distribution of intravenously and orally administered ^{14}C-diazepam and ^{14}C-chlordiazepoxide was studied in mice using the "whole-body" autoradiographic technique. Diazepam accumulates rapidly in the

brain, kidney, liver and myocardium. High concentrations appear rapidly in the gastric mucosa and bile, and consequently in the stomach and intestine.

The distribution patterns of diazepam and chlordiazepoxide were compared. Diazepam is distinguished by an extremely high uptake in the fatty parts of the body. The penetration of chlordiazepoxide in the brain is slower than that of diazepam. The distribution of both drugs and their related metabolites in the brain is quite similar. The white matter and the peripheral nerves retain radioactivity for a long period.

Autoradiographic studies of ^{14}C-diazepam in pregnant female mice demonstrated that there is a slow and rather slight penetration of the radioactive material through the placenta. Analysis of tissue-extracts obtained from mice treated with diazepam yielded at least seven metabolites originating from the labeled material. Rapid metabolic conversion of diazepam was observed.

Half-life times of these products were determined. The metabolites showed a lower elimination-rate than diazepam. N_1-desmethyl-diazepam was found to be the major metabolite in mice.

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CHAPTER VI

KINETICS OF ELIMINATION OF ATARACTIC DRUGS OF THE MEPROBAMATE- AND DIAZEPINE-GROUP IN DOGS

INTRODUCTION

The great interest in the clinical use of ataractic drugs in the relief of anxiety and tensions of psychic and muscular origin promoted the development of large series of neuroleptic drugs of moderate activity : the socalled minor tranquillizers or ataractics.

Meprobamate and homologues have proved their value, and more recently chlordiazepoxide and diazepam were introduced. These are now the most common drugs in clinical and general practice.

The previous reported analytical procedures on estimating propanediols and diazepines in biological medium made repeated analysis of these drugs desirable. The published pharmacokinetic data thusfar were often based on the sum of parent compound and metabolites. The rather rapid metabolism in laboratory animals, as well as in man, made a renewed analytical effort worthwhile.

The purpose of these investigations was to study the fate of ataractics in dogs as an introduction to the study of these drugs in man.

MATERIALS

The following drugs, either cold or ^{14}C-labeled compounds, were investigated : meprobamate, carisoprodol (N-isopropyl-meprobamate, Apesan®), tybamate (N-*n*-butyl-meprobamate, Solacen®), chlordiazepoxide (Librium®) and diazepam (Valium®). Drugs of the meprobamate-group were ^{14}C-labeled in the (unsubstituted) carbamate-group. The diazepines were ^{14}C-labeled in the C_2-position.

To prepare dosages radioactive material was mixed with cold drug to the desired dose in such a way that 0.5–2 μC/kg could be administered to the dogs (TABLE I).

METHODS

Liquid scintillation counting (LSC).

Max. 1.00 ml of plasma was mixed with 1.00 ml of hydroxide of Hyamine® (Packard)

or 1 00 ml of 0 75 M Soluene[R] (Packard) in a countvial The latter mixture had to be warmed for 30 minutes at 50 C When digestion was complete the liquid was mixed with 15 0 ml of scintillation cocktail of Bray (1960)

1 00 ml of protein free supernatant liquid after ultracentrifugation was mixed directly with 15 0 ml of scintillation cocktail Counting was performed in a Nuclear Chicago, Mark I or a Packard 3380 AAA, liquid scintillation counter Corrections were made with the external standard method

Specific activity of the injections was checked before administration by weighing about 1 % of the dose and by subsequent assay by LSC

Gas-liquid chromatography (GLC)

Concentrations of meprobamate, carisoprodol and tybamate in plasma and urine were determined according to the method of Douglas *et al* (1967)

Concentrations of diazepam in plasma were determined according to the method indicated by de Silva *et al* (1964), used by Marcucci *et al* (1968) and described in Chapter 7

Determination of free, plasma water, drug concentration

Free drug concentration was determined by separating the unbound drug molecules from the drug-protein complex by centrifugation in a Christ Omega II 70 ultracentrifuge according to the method previously described (Chapter 2)

1 0 ml of protein free, supernatant fluid was assayed for radioactivity Radioactivity was correlated with mg of parent compound l

Determination of protein concentration

Total protein concentrations were determined with the biuret method Protein spectra were densitometrically assayed after electrophoretical separation on cellulose acetate using amido-black as dye (Clinical Laboratories of Internal Medicine, St Radboud Hospital, Nijmegen)

Animal experiments

Dogs fasted for 24 h, were used in the experiments In a number of animals an implanted polythene catheter was introduced surgically into the aorta via the A iliaca to allow repeated sampling from the unanaesthetized dog (Tholin *et al*, 1965) In other experiments in dogs a polythene catheter was introduced into the aorta via the A femoralis under local anaesthesia This catheter enabled sampling during the first day of the experiment Intravenous injections were given into the V saphena

Preparation of dosage

Injection Drug was solubilized in 10 % ethanol-solution by (2 %) polysorbate 80 and injected as a 10 % suspension

Oral dose Drug dissolved in dichlormethane was mixed with wheat flour and the solvent was evaporated The residue was mixed with minced meat and given orally to the animal

Urine

Every 24-hours urine was sampled in a metabolism-cage Daily, the volume and pH were measured and 20 0 ml of the liquid was adjusted with alkali to pH 7, filled up to 25 0 ml and brought in contact with 25 0 ml of a mixture of chloroform and carbon tetra chloride (1 1) (meprobamate-group) or diethylether (diazepine-group) The rocking-extracting method of Resse *et al* (1964) was used After at least 24 h of rocking 5 00 ml

of each layer was transferred to a countvial and the solvent was evaporated. The residue of the aqueous layer was made up to 1 ml with water, mixed with 15.0 ml of Bray's scintillation cocktail and assayed for radioactivity. The residue of the organic layer was directly mixed with scintillation cocktail.

Results and discussion

Meprobamate-group.

The concentrations of ^{14}C-radioactive drug were followed both in plasma and plasma-water of dogs at various periods after administration of a single dose up to 24 h. After an initial rapid decline, following i.v. injection, the course of the concentration of the radioactive label showed a slight recovery after about 15 minutes. This aspect was more pronounced in the plasma-water concentration of dogs treated with carisoprodol and tybamate. In some experiments the parent compound was simultaneously measured. From Fig. 1 it can be concluded that the metabolites show a much slower elimination rate than the parent drug.

Assay of total radioactivity appeared to be insufficient as an indicator because the drug is rapidly metabolized. Previous reported data of dogs give for meprobamate a half-life ($t\frac{1}{2}$) of 8 h (Hoffman and Ludwig, 1959), for carisoprodol $t\frac{1}{2} = 1$ h (Douglas *et al.*, 1962) and for tybamate $t\frac{1}{2} = 4$ h (Douglas *et al.*, 1966).

The "classical" spectrophotometric method of Hoffman and Ludwig (1959) to determine meprobamate and homologues in biological medium is unable to differentiate between metabolites and parent compound. Considering the short biological half-lives of intact meprobamate, carisoprodol and tybamate compared to their pharmacologically inactive metabolites (Berger and Ludwig, 1964; Berger *et al.*, 1964) this method may give an inflated estimate of the pharmacodynamics.

The described experiments can be based on a simple one compartment model and an exponential elimination (Chapter 1).

The data of the drug and metabolites are summarized in Table I. From this table some conclusions can be made. The *N*-substituted homologues show a shorter $t\frac{1}{2}$ than meprobamate itself. However, the rate of elimination of the radioactive material does not differ in a great extent. The distribution volumes of meprobamate (V_A) and of the sum of drug and metabolites (V_S) can be considered to be about similar. For carisoprodol and tybamate V_A is about two times greater than V_S. These findings can be explained by the higher rate of metabolism and the higher affinity for the lipoid tissues of the body which appear to correspond with the higher lipophilic nature of the drugs.

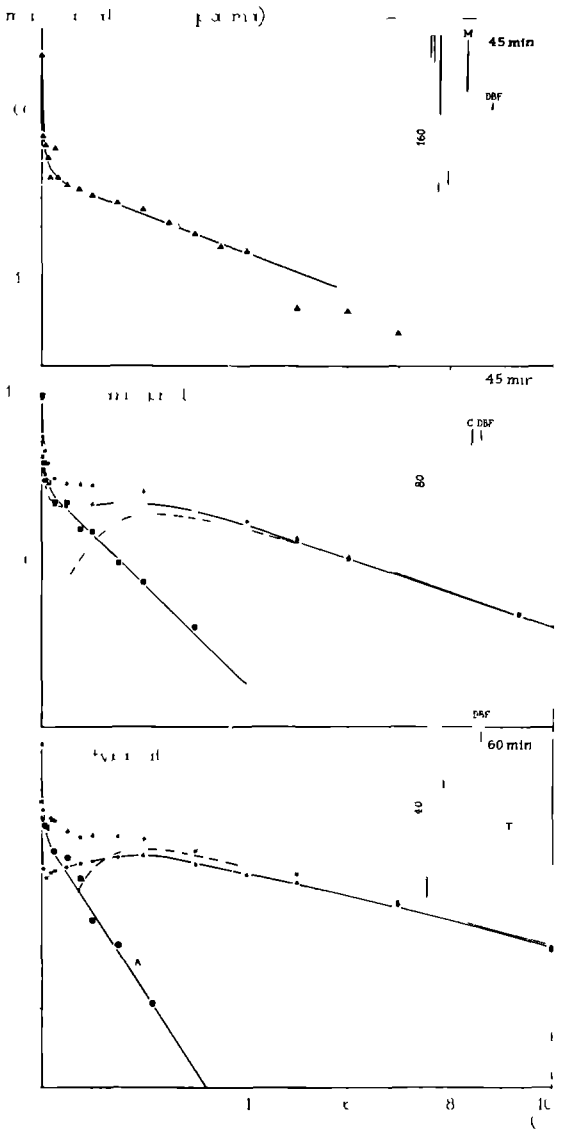

FIG 1

Log concentration (C_A)-time curves of parent meprobamate (M) (▲——— ▲), carisoprodol (C) (■——— ■) and tybamate (T) (● ——— ●) after i v administration of 25 mg kg to dogs *——— * give the course of the concentration of ^{14}C-radioactivity (C_γ) in plasma *——— * give the concentration of ^{14}C-radioactivity in plasma-water (C_{A_F}) The residues of the curves of C_γ and C_A give the curve for the sum of the metabolites (C_M) The insertions give the GLC patterns of the plasma extracts No metabolites can be detected by this analysis procedure Dealkylation of carisoprodol and tybamate cannot be identified in plasma DBF = dibutylphtalate (internal standard)

TABLE I

Drug	Route of Administration	Q_A^0	W_D	D_A^0	Radioactive dose	C_A^0	C_S^0	$t_{\frac{1}{2}A}$	$t_{\frac{1}{2}S}$	V_A	$\triangle A$	V_S	$\triangle S$
		mg	kg	mg/kg	μCi	mg/l	mg/l	h; min	h; min	l	%	l	%
Meprobamate	i.v.	1450	23	63	20		78		5; 42			18.6	93
	i.v.	1000	20	50	15		64		6; 17			15.4	77
	i.v.	545	18	30	25		54		3			10	55
	i.v.	500	23	22	17		55		2; 52			9.1	40
	o.	2000	16.6	120	10		98		6; 05			20	120
	i.v.	800	32	25		40		2; 40		20	63		
Carisoprodol	i.v.	1043	24.5	42.5	30		50		3; 50			21	85
	i.v.	800	22	36.4	13		95		2; 26			8.4	40
	i.v.	650	22	29.5	13.2		74		3; 27			8.8	40
	i.v.	604	24	25.2	26.4	28	44	1	2	21.6	90	13.7	57
Tybamate	i.v.	568	19	30	28	31.5	55	; 43	2; 08	18	95	10.3	54
	i.v.	545	20	27.3	8.4		54		2; 04			10.1	50
	i.v.	660	26	25.4	15.3	33	45	; 39	3; 50	20	77	9.9	38
	i.v.	487	21	23.1	9.0		54		2; 40			9	43
	o.	1750	17.5	100	6.3		39		> 10			45	260
	o.	1800	18	100	10.9		33.5		> 10			53.7	300

Q_A^0 = dose; W_D = weight of dog; D_A^0 = dose/kg body-weight; A = parent drug; S = sum of radioactivity of parent drug and metabolite(s); C^0 = fictive initial concentration; V = fictive volume; $\triangle$ = 100. V/W_D.

The distribution volumes of meprobamate, carisoprodol and tybamate calculated on basis of the plasma concentration do not differ significantly. It is, however, necessary to calculate these values on basis of the plasma-water concentration.

Because it has been shown that the protein-binding capacity of drugs of the meprobamate-group is concentration independent, it is allowed to calculate the distribution volume by multiplying with a factor 100 %/% unbound drug (Chapter 2). The V_A-values for carisoprodol and tybamate should be corrected with a factor $100/57 = 1.8$ and $100/35 = 2.9$, respectively.

In plasma extracts of meprobamate treated dogs no metabolites could be detected (FIG. 1, insertion). Hydroxy-meprobamate is reported as the main metabolite in urine, but cannot be detected with the procedure used (LUDWIG *et al*, 1961).

After carisoprodol treatment no metabolites could be distinguished in plasma. From experimental data obtained in mice, meprobamate was expected to be the majorm etabolite (VAN DER KLEIJN, 1969; Chapter 4). Dealkylation, however, could not be confirmed in dog plasma. It may be explained by the fact that the rate of metabolite (meprobamate) appearance in the plasma is too slow compared to its rate of elimination by subsequent hydroxylation and excretion. Hydroxycarisoprodol was found

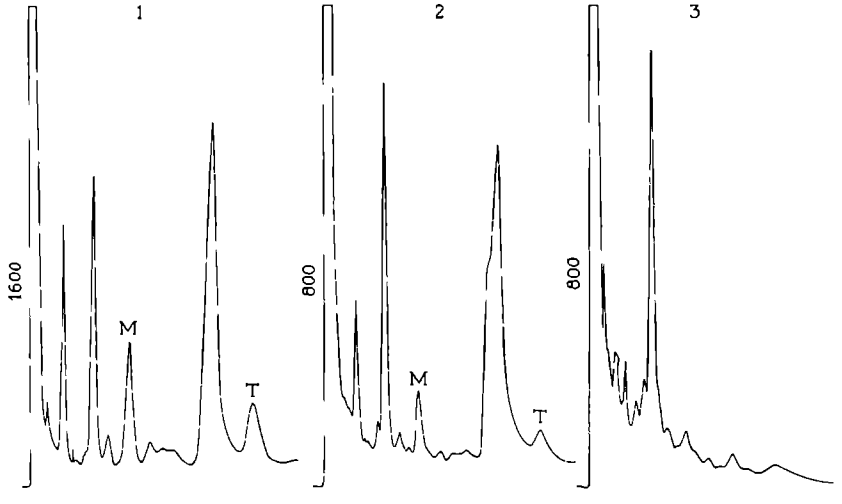

FIG. 2

GLC patterns of daily urine extracts of a dog after a single i.v. dose of tybamate. In the pooled urine of the first two days parent drug and meprobamate as metabolite can be identified in small amounts. The other peaks are due to the drug additive PEG 300.

to be the major metabolite in urine of dogs by EDELSON *et al.* (1965) and DOUGLAS *et al.* (1966).

In the plasma GLC-spectra of the tybamate experiments no metabolites could be detected. In urine, in addition to small amounts of parent drug, meprobamate could be detected following the first two days of the experiment (FIG. 2). Hydroxylation is suggested to be the fastest step in the biotransformation of carisoprodol and tybamate in dogs. 80-95 % of the radioactive material due to meprobamate or homologues could be recovered in the urine after 2-3 days following administration. The major portion of the drug is excreted as highly hydrophilic compounds (FIG. 6).

The simple representation of the data in Table I is only allowed when the initial concentration decline during the first distribution period is neglected. When enough data are determined following rapid injection of the drug an interpretation according to a two compartment open system could have been possible (Chapter 1).

Diazepine-group.

The most specific, non-isotope method to determine chlordiazepoxide in biological medium has been reported by KOECHLIN and D'ARCONTE (1963), but here too, inherent to the procedure the demethylated and deaminated metabolites are simultaneously measured. Gaschromatographic separation of intact chlordiazepoxide has not yet been successful.

Hydrolysis to aminochlorbenzophenon has more disadvantages than the spectrophotofluorometric method since most metabolic transformations take place in the 7-membered ring that is split off during hydrolysis (SCHWARTZ *et al.*, 1968). This last argument holds equally well for diazepam. The rapid appearance in laboratory animals of metabolites with the same analytically detectable moiety, made renewed estimations of these drugs likely, moreover since biotransformation may lead to decrease of pharmacological activity (RANDALL *et al.*, 1965; GARATTINI, 1968).

In the case of chlordiazepoxide, biotransformation can theoretically lead to structures of increased pharmacological activity and increased lipophilic nature. It is doubtful, however, that the rather slow rates of metabolism will have significant influence upon the latentiation kinetics of this drug (SCHWARTZ *et al.*, 1968).

The concentration of ^{14}C-radioactive drug was followed in plasma and plasma-water for 160 h after intravenous and oral administration.

The more complex course of the concentrations of radioactivity in plasma due to the diazepines made analysis of the data into simple first

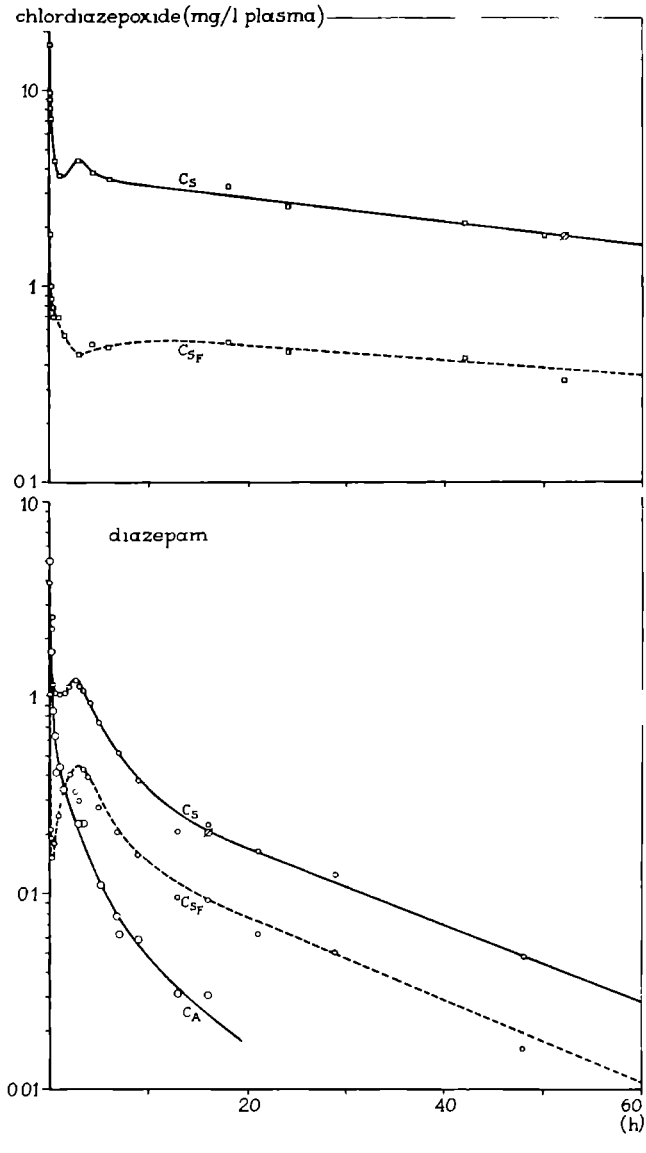

FIG. 3

Log concentration-time curves of chlordiazepoxide (⁻———(·)) and of diazepam (⌒———⌒) after i.v. injections of 3 75 mg/kg and 1 mg/kg, respectively. The course of parent diazepam is much steeper than that of the total radioactivity. The rapid secondary recovery of the plasma and plasma-water ^{14}C-concentration after diazepam injection indicates the rapid metabolism leading to the formation of compounds with smaller fictive distribution volumes and lower binding capacity The elimination of chlordiazepoxide appears to be much slower and the slighter secondary rise of the curves suggests slow biotransformation.

order one or two compartment kinetics impossible. Of chlordiazepoxide in dogs $t_{\frac{1}{2}} = 7$ h was reported (RANDALL, 1960; KOECHLIN *et al.*, 1965) and of diazepam $t_{\frac{1}{2}} = 9$-16 h (SCHWARTZ *et al.*, 1965).

The drug concentration decline was followed by means of their radioactive label situated in the C_2-position. In addition to the parent drug, metabolites were included in the measurements. In some cases diazepam could simultaneously be measured by GLC.

From Fig. 3 it appears that the elimination of chlordiazepoxide is slower than of diazepam. It can be demonstrated that intact diazepam is more rapidly eliminated than its related metabolites.

It may be noted, that after the initial concentration decline a pronounced recovery of the ^{14}C-concentration of diazepam can be observed which is almost absent in the case of chlordiazepoxide. This phenomenon is more pronounced to the plasma-water concentration especially when higher doses are administered (FIG. 3 and 5). After oral administration of chlordiazepoxide biological half-lives of the label are rather equal while large individual variations are observed after intravenous administration (FIG. 4). The concentration of radioactivity reaches its maximum between 2-4 h after administration, and the rising portion of the curve is erratic. Of chlordiazepoxide some data are given in Table II.

Individual differences are pronounced also after oral administration of diazepam while the elimination pattern of radioactivity shows more mutual similarities after i.v. administration (FIG. 5).

In the course of the concentration of radioactivity due to diazepam at least three phases can be distinguished. When the rapid initial decline is neglected the curve can be treated as a bi-exponential curve, and data on basis of a two compartment system are summarized in Table III (compare Chapter 1). There are, however, insufficient data for exact analyses of the parameters. From whole body autoradiographic distribution studies evidence is obtained that the drug is rapidly and extensively exchanged with the lipoid tissues of the body. The clearance ratio k_{12}/k_{21} in a two compartment system will be very great. Since this ratio is unknown, it is not possible to predict the concentration course in the peripheral compartment nor the fictive distribution volume. No simple relations are observed between various doses and plasma concentration (FIG. 4 and 5).

In the plasma spectra of diazepam treated dogs desmethyldiazepam appears after approximately 1 h after injection in the region of the diazepam peak. Oxydiazepam and oxazepam can hardly be detected in the spectra.

About half of the dose is excreted with the urine (FIG. 6). The rest is excreted via the feces (SCHWARTZ *et al.*, 1965).

Theoretical model for interpretation of the secondary recovery of the log ^{14}C plasma concentration-time curve after intravenous injection.

The use of a radioactive isotope as tracer for a drug in the body may give difficulties when the label passes through all metabolic pathways.

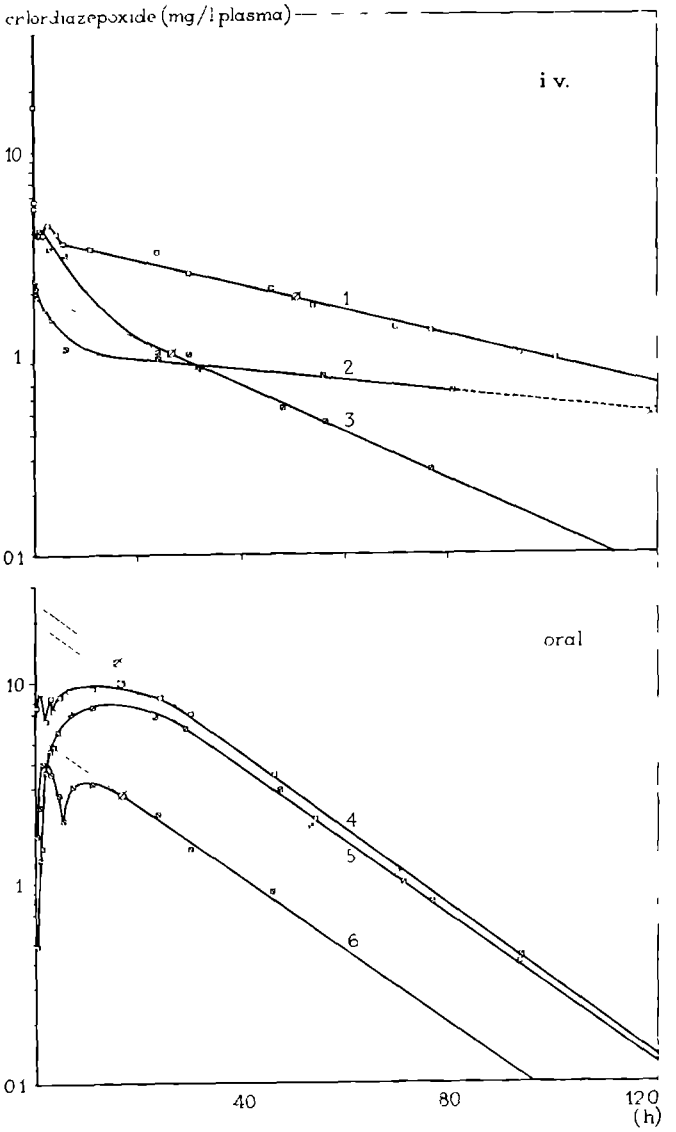

FIG. 4

Log. concentration-time curves of ^{14}C-chlordiazepoxide (1)——(6) following i.v. and oral administration of 1) 3.75, 2) 2.2, 3) 1.1, 4) 10, 5) 8.3 and 6) 5 mg/kg respectively.

TABLE II

Drug	Route of administration	Q_A^0	W_D	D_A^0	^{11}C	L_{2S}	$t\frac{1}{2}$
		mg	kg	mg/kg	μCi	mg/l	h
Chlordiazepoxide	i.v.	90	24	3.75	22.4	3.8	50
	i.v.	50.6	23	2.2	21.8	2	26.5
	i.v.	22.3	20	1.11	10.4	1.05	100
	oral	210	21	10	34.9	25	16
	oral	166	20	8.3	20.8	20	16.5
	oral	114	23	5	23.3	5.6	16

L_2 = intercept of second phase of log concentration-time curve; $t\frac{1}{2}$ = half-life of flat portion of the bi-exponential curve.

The appearance of metabolites into the plasma after intravenous administration can be interpreted by the equation which is used to describe absorption of drugs versus elimination (DOST, 1968; Chapter 1, equation 14).

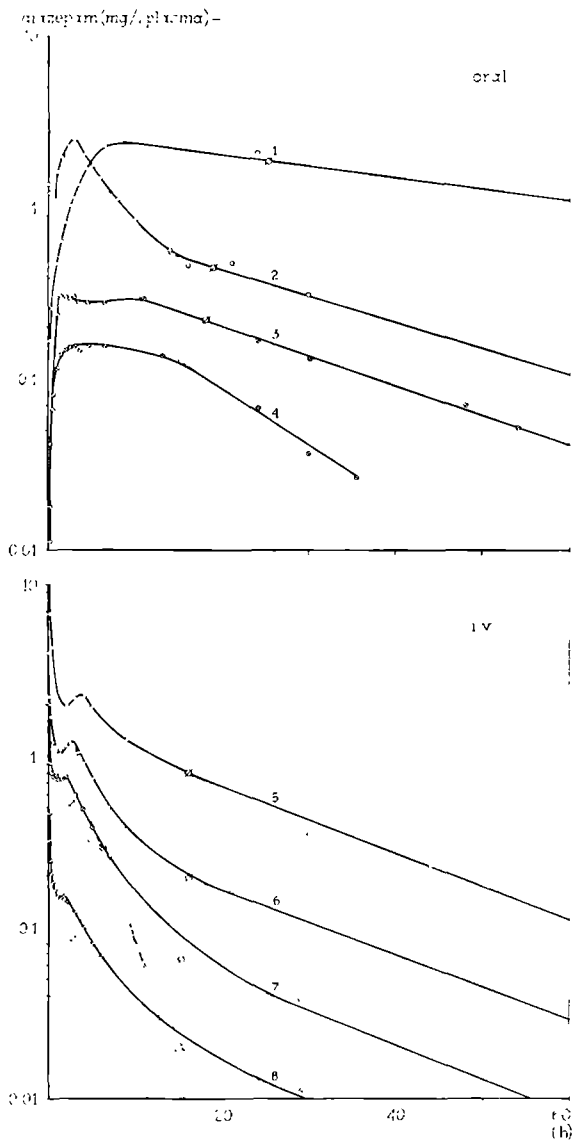

FIG. 5

Log. concentration-time curves of ^{14}C-diazepam (○———○) following i.v. and oral administration of 1) 6.7, 2) 4.2, 3) 1.4, 4) 3, 5) 2.1, 6) 1, 7) 0.88 and 8) 0.36 mg/kg.

TABLE III

Drug	Route of administration	Q_A^0	W_D	D_A^0	L_1	L_2	V_1	$\triangle A$	$t_{\frac{1}{2}1}$	$t_{\frac{1}{2}2}$
		mg	kg	mg/kg	mg/l	mg/l	l	%	h; min	h; min
Diazepam	i.v.	58.4	23.5	2.06	3.5	1.6	11.45	50	1 ;50	16
		26.5	27	0.98	2.0	0.4	11.04	41	2; 10	16; 10
		22	25	0.88	1.05	0.13	18.06	72	2; 40	15; 15
		7	19	0.36	0.17	0.04	33.3	175	2; 50	
	oral	120	18	6.7		2.8				25
		80	19	4.2		0.9				19
		32	23	1.4		0.45				18
		51	17	3		0.40				9

L_1 = intercept of residue of biphasic curve with ordinate; L_2 = intercept of second phase of log concentration-time curve; $t_{\frac{1}{2}1}$ — half life of residual curve; $t_{\frac{1}{2}2}$ — half-life of flat portion of the bi-exponential curve.

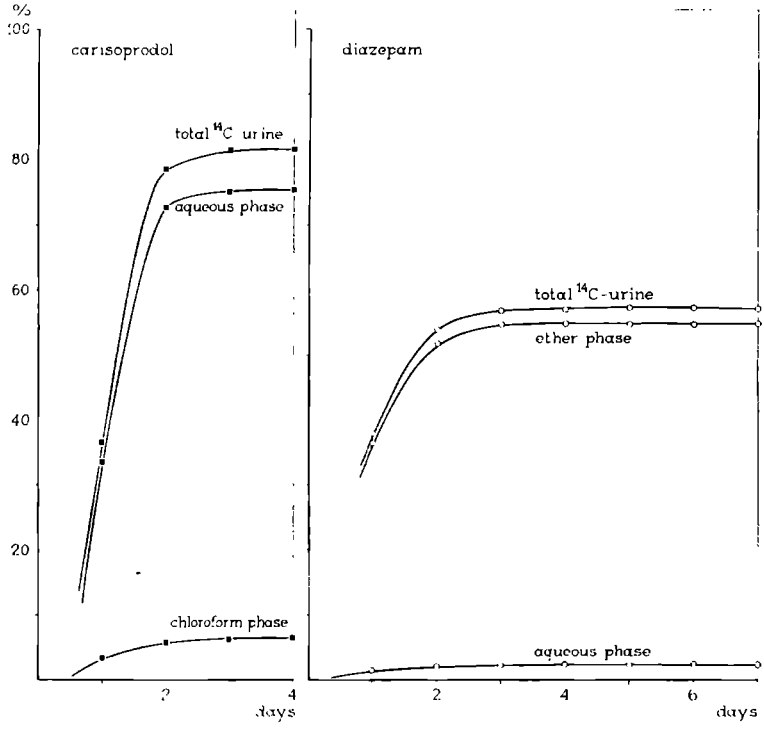

FIG. 6

Cumulative urinary excretion patterns of carisoprodol and diazepam. About 81 % of the administered carisoprodol can be recovered from the urine. The major portion is excreted as water-soluble material. Of diazepam about 57 % of the dose can be found in the urine. The major portion of the radioactive material can be extracted with ether. It is suggested that the conjugates of the main metabolite in urine: oxazepam, (RUELIUS *et al.*, 1965) are rather unstable.

This equation reads for drug and metabolite together:

$$C_s^t = (Q_A^0/V_A) \cdot e^{-t/\tau} + (Q_A^0/V_M) \cdot [\tau_{2M}/(\tau_{2M} - \tau)] \cdot [k_{M3}/(k_{M3} + k_{13})] \cdot (e^{-t/\tau_{2M}} - e^{-t/\tau})$$

where: τ = the time constant for elimination of drug A, which is considered to be equal to the time constant of metabolite appearance in the plasma, τ_{2M} = the time constant of elimination of the metabolite, and C_S^t = molar concentration of radioactive material.

In the group of ataractic drugs elimination is predominantly controlled by biotransformation. Since the lipophilic nature of the compounds prevents their renal excretion, k_{13} can be neglected. The factor $k_{M3}/(k_{M3} + k_{13})$ may thus be considered to be equal to 1. τ_{2M} can be derived

from the last phase of the plasma curve of the radioactive label when the parent drug is considered to be eliminated. When the distribution volumes of parent compound and metabolite are known then the time constant of the parent compound can be determined. Fig. 7 shows two examples where the distribution volume of the metabolite (V_M) is varied.

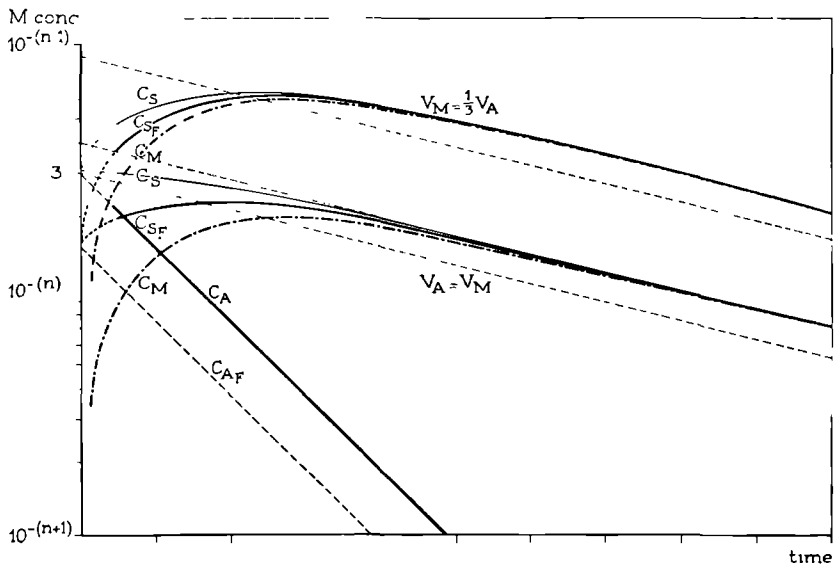

FIG. 7

Theoretical log. concentration-time curves calculated for two different fictive distribution volumes of the metabolites. C_S = plasma concentration of sum of metabolites (M) and drug (A) together; C_{SF} = plasma-water concentration of drug and metabolite together; C_A — concentration of parent drug in plasma; C_{AF} — concentration of parent drug in plasma-water, protein-binding capacity drug: 50 %, protein-binding capacity metabolite: 0 %; $C_A^0 = 3 \times 10^{-n}$ M; $\tau_A = \tau_{1M} = 1.44$ h; $\tau_{2M} = 5.76$ h.

Biotransformation of meprobamate, carisoprodol, tybamate and diazepam leads to compounds of reduced lipophilic nature expressed as partition coefficients and reduced protein-binding capacity. The progressively decreasing protein-binding capacity of the ^{14}C-label during the course of the experiment accentuates the steeper rise in the C_{SF}-curve (FIG. 1, 3 and 5).

These models allow at least the qualitative interpretation of experiments when drug concentrations are followed by an unselective analysis procedure.

The following conclusions can be made for the dogs:

1. Drugs of the meprobamate-group are rapidly metabolized and the parent drugs show higher elimination rates than their related metabolites.

2. Carisoprodol and tybamate are predominantly metabolized to hydroxy-carisoprodol and hydroxy-tybamate respectively, and excreted as such or as conjugates with glucuronic acid (DOUGLAS *et al.*, 1962, 1966). Faster elimination corresponds to higher lipophilic nature.

3. After tybamate treatment a small amount of parent drug is excreted and meprobamate can be recovered in urine (FIG. 2), (DOUGLAS *et al.*, 1966).

4. The concentration time curve of ^{14}C-chlordiazepoxide after intravenous administration differs markedly in various dogs. After oral administration, a closer resemblance in elimination rate is observed. However, fluctuations in reaching the maximum plasma concentration, that is reached between 2-4 h after administration, suggest retarded absorption of the drug from the gastro-intestinal tract or abundant enteral recirculation of the drug and/or the metabolites.

No simple relations between dose and maximum plasma concentration are observed.

5. Diazepam and its related metabolites show the same individual variations as chlordiazepoxide. After i.v. administration some mutual resemblances in the concentration course of ^{14}C-diazepam is observed. The metabolites show much slower elimination than the parent compound. Biotransformation is reported to yield compounds of decreased activity and of lower lipophilic nature.

No simple relations between dose and plasma concentrations were found. The multiphasic elimination curves require more data and study to be interpreted in terms of two or multi-compartment pharmacokinetics.

SUMMARY

Plasma concentrations of meprobamate, carisoprodol, tybamate, chlordiazepoxide and diazepam were studied in dogs after i.v. and oral administration. The ^{14}C-labeled compounds were determined by liquid scintillation counting giving the sum of drug and metabolites. In some cases the parent drug was simultaneously assayed gaschromatographically. Metabolites appeared to have longer biological half-lives than the parent compound. The concentration course in plasma of drugs of the meprobamate-group is interpreted in simple one-compartment kinetics. Chlordiazepoxide- and diazepam-treated dogs showed considerable individual variations. No simple relation between dose and plasma concentrations could be established. The multiphasic log concentration-time curves of diazepam require further study.

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CHAPTER VII

PHARMACOKINETICS OF DIAZEPAM IN MAN

INTRODUCTION

Diazepam (Valium®) is a frequently used ataractic in the clinical and general practice of light neuroses and psychic and muscular tensions. Since, unfortunately, only subjective criteria in the benefit of the therapy can be made, a more objective approach is desired on the basis of plasma concentrations.

Diazepam levels have been studied in man and laboratory animals. Dealkylation, hydroxylation and subsequent conjugation were reported as the major metabolic pathways. Desmethyldiazepam appeared to be the major metabolite in plasma. Free and conjugated oxazepam was predominantly found in urine (RUELIUS *et al.*, 1965; SCHWARTZ *et al.*, 1965; DE SILVA *et al.*, 1966). Following a single oral dose of ^{3}H-diazepam a multiphasic concentration decline was reported by SCHWARTZ *et al.* (1965) with an overall half-life of about 3 days after a faster initial phase. From the work of DE SILVA *et al.* (1966) it appeared that diazepam shows a half-life of 30 h after an initial faster period following a single oral dose of of 10 mg diazepam, but of 2-3 days after repeated doses. The $t\frac{1}{2}$ of desmethyldiazepam appeared to be much longer. The elimination of diazepam thus seemed to be greatly dependent on the preceding dosage history. Incomplete distribution after a single dose over all possible compartments was used to explain the erratic concentration-time course of the drug.

RIEDER and RENTSCH (1968) reported that desmethyldiazepam and diazepam were the major metabolites of medazepam (C_2-desoxydiazepam) in man. From their data it may also be concluded that desmethyldiazepam is much slower eliminated than diazepam. From experiments in mice and dogs longer half-lives of the main metabolites other than that of the parent compound were found (VAN DER KLEIJN, 1969).

Since biotransformation affects the pharmacological activity only quantitatively (RANDALL *et al.*, 1965; GARATTINI, 1968), differences in biological half-life may have consequences during chronic treatment.

It is more reasonable to derive pharmacokinetic parameters from concentration levels after chronic treatment, when accumulation has led to a plateau level and a steady state is established. The purpose of this investigation was to study pharmacokinetics of elimination and metabolism of diazepam in man on the basis of :

a. plasma concentration decay curves of drug and metabolites after a single oral dose of 30 mg of diazepam, b. plasma levels during chronic therapy with oral doses of 10 mg, 3 times daily, and c. plasma concentration decay curves of drug and metabolites after completion of the therapy.

MATERIALS

Instrument.

The F & M model 402 dual column gaschromatograph (GLC) equipped with a ^{63}Ni-electron capture detector (ECD) and a flame-ionization detector (FID) combined with a 1 MV Moseley recorder was employed.

Columns : 3 meter glass and copper tubes (internal diameter 2.8 mm) packed with 3.8 % UC-W 98 methyl silicone on 80 100 mesh Diatoport S (Packing . Hewlett-Packard, Amsterdam); carrier : nitrogen; purge : argon-methane (90 : 10).

Instrument settings.

Temperature : column : 250° C; injection port : 330° C; detector block : 290° C; auxiliary heating : 300° C.

Gas-flow rate : ECD : N_2 75 ml/min; argon-methane 200–220 ml/min, measured at detector outlet.

FID : N_2 2.2 – 50 ml/min; H_2 3.0 – 40 ml/min; O_2 4.5 – 500 ml/min.

Pulse interval : 50–150 μsec; amplification : range 1–10; attenuation 2–16; chart speed : 0.25–0.5 inch/min.

METHODS

GLC procedure.

2 00 ml of a heparinized plasma sample containing diazepam and derivatives is transferred into a 40 ml glass-stoppered centrifuge tube; 2.0 ml 0.2 M phosphate buffer solution (pH – 6.8) and 10 ml diethyl ether (reagent grade) are added. The stopper is sealed with one drop of water. The tubes are thoroughly shaken for 10 min. The layers are separated by centrifugation. The organic layer is transferred into another 40 ml glass-stoppered tube. The sample is re-extracted with ether. The combined ether phases are evaporated in a waterbath at 30° C by an air current. The residue is rinsed with dried ether to the bottom of the tube and re-evaporated. The residue is dried in a vacuum dissiccator over anhydrous $CaCl_2$.

100 μl of *n*-hexane containing 5 μg 2-N-benzyl-amino-5-chlor-benzophenone (BACB) is added. The residue is dissolved for 10 min; 1–4 μl of the liquid is analyzed. Concentrations are correlated with the ratios of the peak areas of compound and internal standard calculated from peak height, and width at half height.

GLC injections containing a 2 ng compound corresponding to a 25 ng/ml sample can accurately be determined. Samples containing 10 ng/ml sample can qualitatively be

identified. Linearity is determined for 50–1000 ng diazepam/ml sample and 50–1000 ng desmethyldiazepam/ml sample (FIG. 1).

The retention times of oxazepam, diazepam, desmethyldiazepam and BACB account for about 6, 8, 9.5 and 17 min respectively.

Determination of protein.

Total protein content was determined by the biuret method. Protein spectra were densitometrically measured after electrophoretical separation using amido-black as dye (Clinical Laboratories of Internal Medicine, St. Radboud Hospital, Nijmegen).

Determination of protein binding.

Separation of free drug and drug-protein complex was achieved by the ultracentrifuge method as previously described (VAN DER KLEIJN, 1969; Chapter 2).

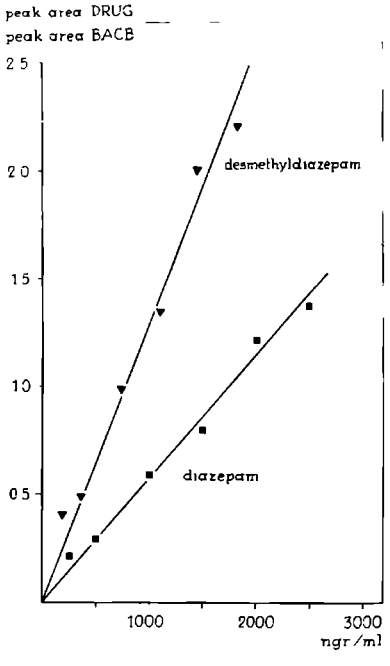

FIG. 1

Calibration curves of diazepam (●——●) and desmethyldiazepam (▼——▼) in human plasma. Pulse interval 150 μsec.

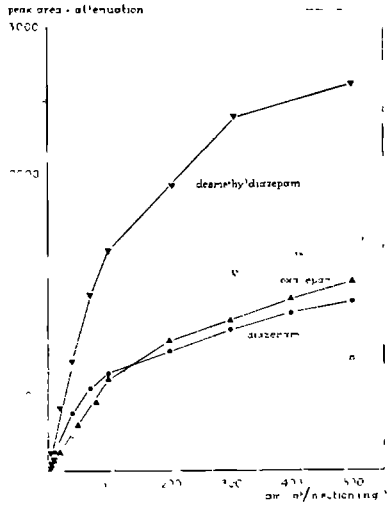

FIG. 2

Relationships between amount and response for desmethyldiazepam (▽, ▼), diazepam (○, ●) and oxazepam (△, ▲) at pulse intervals of 150 μsec (———) and 50 μsec (— — —).

RESULTS AND DISCUSSION

Method.

The mean therapeutic plasma concentration levels of diazepam were found to range from 0.4-2 μg/ml. For the determination of these low

concentrations in the presence of metabolites and biogenous compounds a sensitive and specific method is necessary.

A method proposed by DE SILVA *et al.* (1964) enabled the determination of diazepam levels in plasma after hydrolysis in acid medium in the nanogram range. Determination of the intact drug on a 2 feet 5 % silicone DC 11 column with ECD was advised against because of its slight separation capacity. Although the hydrolysis method is recommended for the simultaneous determination of diazepam and its metabolites in the same sample, the extraction and re-extraction procedure is insufficient for quantitative extraction of oxazepam and oxydiazepam. Desmethyldiazepam was found to be the major metabolite in plasma. The procedure is thus useful to plasma where no other metabolites were identified by the authors.

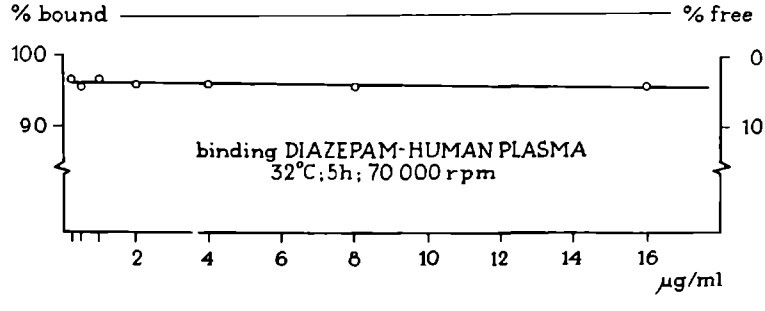

FIG. 3

Protein-binding capacity of diazepam in human plasma measured according to the ultracentrifuge method.

MARCUCCI *et al.* (1968) returned to the analysis of intact drug by GLC on a Silicone gum column of 2 m length which allowed the clean separation of oxazepam, diazepam, desmethyldiazepam and oxydiazepam successively. BACB was used as an internal standard.

In the experiments reported here a 3 m column was used and the flow rate of the carrier gas was increased to improve the dissolving power of the column. Electron capture detection was preferred over FID for performance of cleaner separation and about a 50-fold increase in sensitivity. ^{63}Ni-ECD appeared to be the most sensitive for N-desmethyldiazepam. Diazepam could be detected with sensitivity reduced to 1/3 and oxazepam to 1/2. Linearity of response was achieved over the expected concentration range in the plasma (FIG. 2). The method described is simple and rapid. Diazepam and its related metabolites (FIG. 5), nitrazepam and medazepam, can separately be assayed (nitrazepam only by FID).

In all experiments oxazepam and oxydiazepam could not be identified in plasma extracts (FIG. 6). Free and conjugated oxazepam are reported as the major metabolites in urine (RUELIUS *et al.*, 1965; SCHWARTZ *et al.*,

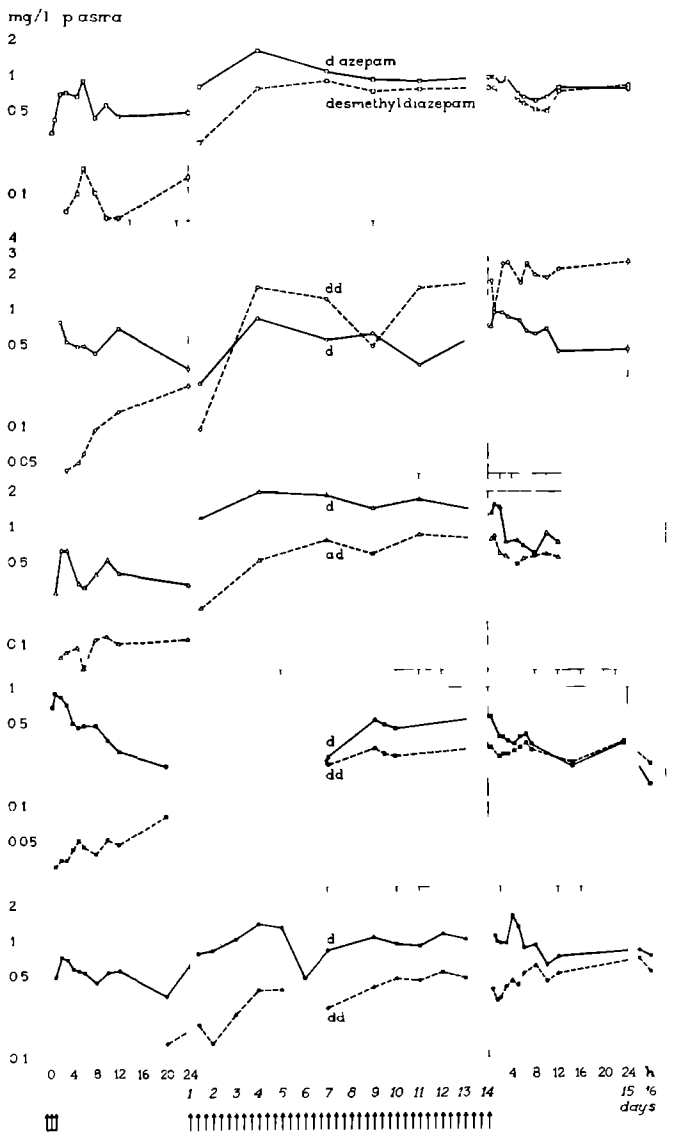

FIG. 4

Log concentration-time curves of diazepam and its major metabolite N-desmethyldiazepam after an initial dose of 30 mg followed by chronic treatment of 3 daily doses of 10 mg diazepam orally in 5 patients.

1965). It may be concluded that elimination by renal excretion is a faster process than hydroxylation and subsequent conjugation.

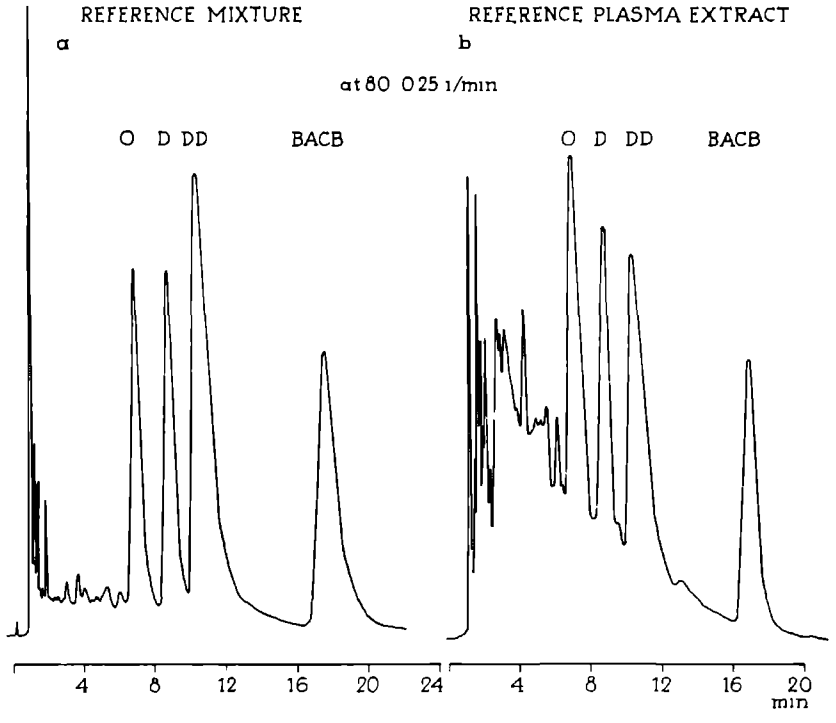

FIG. 5

GLC patterns.

A. Separation of oxazepam (O), diazepam (D), desmethyldiazepam (DD) and 2N-benzyl-amino-5-chlor-benzophenone (BACB) in hexane.

B. Separation of O, D, DD and BACB after extraction from plasma.

Concentration-time course of diazepam in human plasma.

From experiments in dogs it appeared that interpretations of diazepam curves in simple two-compartment kinetics are not possible (Chapter 6).

Analysis of plasma extracts of diazepam-treated patients after a single dose seems to show great individual variations in maximum concentration, in time necessary to reach the maximum, and in metabolite appearance rate.

In the first concentration decay period fluctuating values are observed. The $t_{\frac{1}{2}}$ showed variations between 10 and 40 h (FIG. 4). The rather long half-life of diazepam gave rise to accumulation. After about 4 days the accumulation plateau is reached. The $t_{\frac{1}{2}}$ and the accumulation plateau

concentration ($\bar{C}_{Apl}$) allows the calculation of the fictive distribution volume of diazepam at the steady state of the drug concentration in all possible compartments.

According to VAN ROSSUM and TOMEY (1968):

$$C_{A_{pl}} = 1.44(Q_A^0/V_f)(t_{\frac{1}{2}}/\Delta t)$$

When $\bar{C}_{Apl}$ is determined, the dosage interval $\triangle t$ is known, $t_{\frac{1}{2}}$ is known, then the fictive distribution volume can be calculated, under the assumption that absorption from the gastro-intestinal tract is complete. The values for V_f are given in Table I.

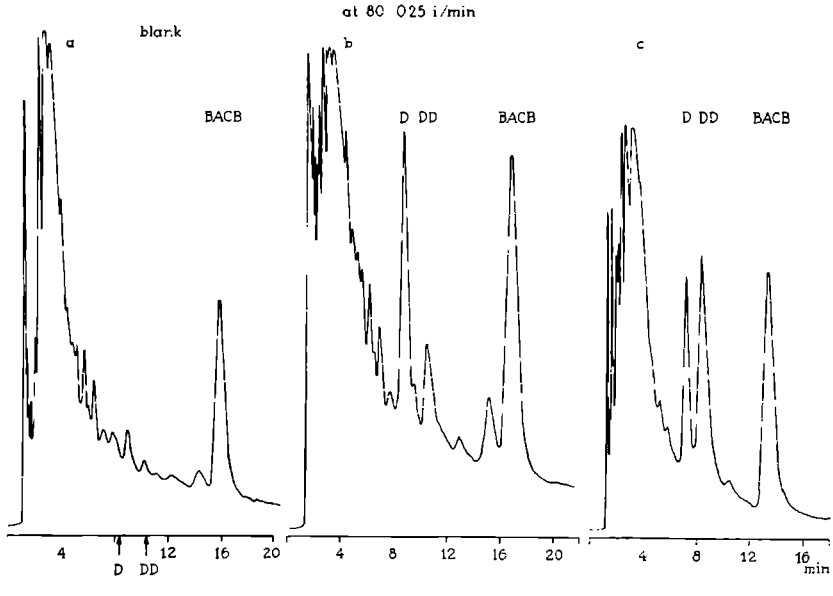

FIG. 6

GLC patterns of plasma extracts.

A. Blank; B. 5 h after the first dose; C. 7½ h after the last dose following chronic treatment during 14 days.

Since only the free concentration is involved in the exchange of drugs with other tissues, the fictive volume has to be calculated from the plasma-water concentration (KRÜGER-THIEMER, 1968).

Estimation of protein binding in outdated plasma shows no concentration dependence over the therapeutically possible concentration range (FIG. 3). So the correct value for V_f can simply be found by multiplication with 1/fraction unbound drug (1/0.04 = 25).

Fig. 4 shows the concentration-time course of diazepam and des-

TABLE I

Patient	Symbol	Sex	Age	Body Weight	$\bar{C}_{Apl}$	$\bar{C}_{Mpl}$	1 $t_{\frac{1}{2}A}$	2 $t_{\frac{1}{2}A}$	2 $t_{\frac{1}{2}M}$	1 V_{fA}	2 V_{fA}	2 V_{fM}	1 V_{fAfree}	2 V_{fAfree}	t_{acA}	$t_a t_M$
			years	kg	mg/l	mg/l	h	h	h	l	l	l	l	l	days	days
N	△	♂	51	60	1.7	0.8	—	—	>50	—	—	>110	—	—	4	5
K	○	♂	20	63	1.4	0.6	>30	8.7	>50	>40	11.2	>150	>1000	280	4	—
M		♂	35	70	1.0	0.8	—	12.3	15	—	22.1	> 34	—	520	4	4
D_1	●	♂	53	58	1.0	0.5	21.5	—	>50	39	—	>180	1000	—	4	5
D_2	■	♂	45	64	0.5	0.28	12.3	23	>50	44.3	85.5	>320	1100	2100	—	—

$\bar{C}_{pl}$ = average plateau concentration. A = parent drug (diazepam), M = metabolite (desmethyldiazepam). 1 = first period after single dose of 30 mg diazepam. 2 = period after last dose of 10 mg diazepam. V_f = fictive volume calculated from plasma concentration. V_{free} = fictive volume calculated from plasma-water concentration. t_{ac} = time of reaching plateau level.

methyldiazepam in five patients. It may be noted that it is difficult to derive exact pharmacokinetic parameters from the observed data. The biological half-life and hence τ cannot be determined exactly. On the basis of highly approximated $t_{\frac{1}{2}}$ values (TABLE I) the fictive distribution volume is calculated. It appears that V_f values show capricious variations. From t_{ac}, the time in which the plateau level is reached it can be calculated that $t_{\frac{1}{2}}$ of diazepam is approximately 1 day and $t_{\frac{1}{2}}$ of desmethyldiazepam is about 30 h ($t_{\frac{1}{2}} = t_{\frac{1}{2}ac} = 1/4\ t_{ac}$). t_{ac} of diazepam shows much resemblance in 4 patients.

It may be clear that diazepam shows a great fictive distribution volume (V_f) which will very slowly be cleared. The elimination occurs predominantly by biotransformation since no intact diazepam could be recovered from urine. Although diazepam and desmethyldiazepam show long biological half-lives the accumulation level becomes not very high. The rapid exchange with other compartments and the great fictive distribution volume make interpretations of plasma concentration levels soon doubtful. Because of the high clearance ratio k_{1N}/k_{N1} plasma concentrations will never synchronically follow the fluctuations of drug concentrations in for distribution and action specific regions containing high amounts of drug (Chapter 1). Therefore it is not excluded that rather small variations in plasma concentration will have great consequences in a peripheral compartment.

The high uptake of diazepam in adipose tissue (VAN DER KLEIJN, 1969) may also give rise to individually varying extension of the fictive distribution volume. The fictive distribution volume is generally considered to be constant for one individual and drug, independent of the applied dose (DOST, 1968).

It is questionable if this constant value will be reached for diazepam and other lipophilic drugs with high specific regional distribution at low dosages. The great capacity of this volume may explain the great inter- and intra-individual variations.

The weakly basic nature of diazepam ($pK_a = 3.3$) and its high lipid solubility can be used to explain the abundant gastroenteric circulation and its strong binding capacity to macromolecules. These factors can contribute to a sustained reabsorption of the drug. Therefore, composition of diet, condition of gastrointestinal tract etc. will be important factors in the absorption of the drug. In addition to the lipophilic parts of the body, the gastrointestinal tract may function as a drug depot, thus increasing the fictive distribution volume.

After a single dose the action of diazepam is based on the presence of parent drug. However, after chronic treatment the action must be con-

sidered to be due to diazepam and desmethyldiazepam together. The diazepam levels no longer reflect the therapeutic concentration. Although desmethyldiazepam is reported to show decreased and slightly modified activity in laboratory animals (RANDALL *et al.*, 1965; GARATTINI, 1968), the contribution to the therapeutic effect of this accumulating metabolite cannot be neglected.

Further investigations over longer medication periods in man are necessary to clarify the intricate pharmacokinetics of this extensively used drug.

Casuistical observations.

a. In one patient (*H*) only very small amounts of desmethyldiazepam could be detected in plasma during the first three days of medication. Due to undesired clinical reaction to the therapy intake of diazepam was stopped.

b. An ambulant patient was examined for suspected overdosage of diazepam. From the plasma GLC pattern it was possible to conclude that the concentration of diazepam was not higher than the normal therapeutic level.

From the amount of desmethyldiazepam, besides diazepam, it could be concluded that the drug had been taken for at least one week.

The simultaneous measurement of diazepam and desmethyldiazepam in ambulant patients allows the identification of either occasional or chronic intake or acute abuse.

c. Recently an alcohol addicted patient (*B*) was treated with diazepam. Considerably shorter biological half-lives could be found than in the previously described patients. Traces of desmethyldiazepam could only be detected after two days following the initial dose of 45 mg diazepam. The dosage schedule was 3 d. d. of 15 mg.

SUMMARY

Pharmacokinetics of diazepam (Valium®) and its major metabolite were studied in plasma from psychiatric patients on fixed dosage regimens. For this purpose GLC with ^{63}Ni-electron capture detection of the intact compounds was used.

As a consequence of the regimens an accumulation plateau level of the drug and its primary metabolite, N_1-desmethyldiazepam, was achieved, which enabled the calculation of the fictive distribution volume (V_f) of the drug and its biological half-life ($t_{\frac{1}{2}}$). Large individual variations were

demonstrated. Plateau levels of diazepam and desmethyldiazepam were reached after about 4-5 days indicating almost equal biological half-lives.

Distribution over a large fictive volume, negligible excretion, predominant elimination by biotransformation and storage in depots in adipose tissue and gastrointestinal tract are assumed to be responsible for the long half-life and the individual variations.

A method is described for the quantitative determination of diazepam and its major metabolites in biological medium in the therapeutic concentration range. The method is also valuable for medazepam (Nobrium®). Nitrazepam (Mogadon®) can be determined by using a flame-ionization detector. Plasma GLC patterns enable the identification of occasional or chronic intake or acute abuse.

The clinical studies on diazepam were conducted by: Dr. G. P. J. A. VAN LIER, Psychiatrisch Centrum "St. Servatius", Venray, Dr. G. C. DE RUYTER DE WILDT, Psychiatrisch Ziekenhuis "Brinkgreven", Deventer, and Dr. J. VAN DER LINDEN, Psychiatrisch-Neurologische Kliniek, "St Radboud" Ziekenhuis, Nijmegen.

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SAMENVATTING

(SUMMARY IN DUTCH)

Het overvloedige en nog steeds toenemende gebruik van kalmerende middelen in de klinische en algemene praktijk is de aanleiding geweest tot het verzamelen van gegevens betreffende de farmacokinetische, analytisch chemische en klinisch farmaceutische aspecten van enkele geneesmiddelen welke veelvuldig toegepast worden bij neuroses, angsten, agressiviteit en spanningen. Tevens worden deze middelen om hun specifieke centrale spierrelaxerende en anticonvulsieve eigenschappen toegepast in de kliniek.

Vaak is de keuze van het juiste psychosedativum, meer dan bij welke andere groep van geneesmiddelen, afhankelijk van subjectieve overwegingen zowel van de behandelende arts als van de patiënt.

Naast de reeds gedurende meer dan een halve eeuw gebruikte barbituraten, bromiden, zuurureïden en chloral-derivaten is omstreeks 1950 met de introductie van meprobamaat een ontwikkeling ingezet van geneesmiddelen, die ter onderscheiding als psychosedativa gekenschetst worden. Deze middelen onderscheiden zich van de „klassieke" sedativa doordat zij een kalmerende werking vertonen bij doses die nog geen sufheid of slaperigheid veroorzaken. De specifieke werking bij angst- en conflictsituaties, die ook bij proefdieren aantoonbaar is, maakt dat deze middelen als ataractica een eigen groep vormen. In de Amerikaanse literatuur worden deze verbindingen als minor tranquillizers aangeduid, welke onderscheiden kunnen worden van de major tranquillizers, waarvan de fenothiazines (chloorpromazine) en de butyrofenonen (haloperidol) de voornaamste vertegenwoordigers zijn. Deze als neuroleptica aangeduide geneesmiddelen worden voornamelijk bij de behandeling van psychoses gebruikt.

Het doel van het onderzoek was de bestudering van de distributie, biotransformatie en eliminatie van de geneesmiddelen van de meprobamaat- en de diazepine-groep.

Ataractica vertonen in het algemeen een grote affiniteit voor de vetachtige weefsels van het lichaam, waardoor slechts geringe concentraties in de voor chemische analyse toegankelijke lichaamsvloeistoffen aan-

wezig zijn. De dikwijls moeilijk analyseerbare verbindingen en de door de biotransformatie gelijktijdig optredende chemisch nauw verwante, doch farmacologisch van elkaar verschillende metabolieten hebben bij eerder onderzoek niet altijd de juiste gegevens opgeleverd.

Het veelal langdurig gebruik van deze ataractica maakt het wenselijk inzicht te hebben in de biologische verblijftijd welke gekarakteriseerd wordt door half-waardetijden ($t_{\frac{1}{2}}$) of tijdskonstantes (τ), door klaringskonstantes (k) en door het verdelingsvolume (vanwege het principieel ontbreken van gelijkenis met de fysiologische verdelingsruimten aangeduid als fictief verdelingsvolume, V_f).

In hoofdstuk 1 wordt een algemeen overzicht gegeven van de factoren, die een rol spelen bij de resorptie, distributie, eliminatie en biotransformatie van geneesmiddelen.

In hoofdstuk 2 is de nadruk gelegd op enkele facetten, die invloed kunnen hebben op de kinetische processen in het lichaam, zoals eiwitbinding en fysisch-chemische eigenschappen. De uitwisseling met andere weefsels wordt mede bepaald door de concentratie in het plasmawater. Het is belangrijk de omvang en de aard van de eiwitbinding te kennen.

In hoofdstuk 3 is een beschrijving gegeven van de techniek van de macroautoradiografie. Deze techniek maakt het mogelijk de verdeling van water- en vet-oplosbare verbindingen zonder gevaar voor verstoring van het biologisch milieu in intacte proefdieren te vervolgen. Een geringe variatie maakt deze techniek geschikt voor het bestuderen van de verdeling van vluchtige verbindingen en/of van vluchtige radioactieve afbraakproducten. Dank zij deze methode kunnen geneesmiddelen ook in voor de analyse ontoegankelijke weefsels en organen gevolgd worden. Combinatie met de klassieke extractie-analyse van weefselhomogenaten maakt het mogelijk het aandeel van de metabolieten op het verdelingspatroon te leren kennen.

In hoofdstuk 4 is voor meprobamaat, carisoprodol (Solacen®) en tybamaat (Apesan®), en in hoofdstuk 5 voor diazepam (Valium®) en chloordiazepoxide (Librium®) de kinetiek van verdeling en metabolisme bij muizen weergegeven. Met behulp van de macroautoradiografie zijn verdelingspatronen verkregen na verschillende tijden en wijzen van toediening. Door toepassing van chromatografische scheidingen en stralingsmeting zijn de oorspronkelijke stoffen en de metabolieten kwantitatief gevolgd. Fysisch-chemische eigenschappen, b.v. uitgedrukt als verdelingscoëfficiënten tussen een organisch oplosmiddel en water, zijn in verband gebracht met de graduele verschillen van penetratie in de lipofiele delen van het lichaam. Grote lipofiliteit van het geneesmiddel blijkt samen te gaan met een snelle penetratie in b.v. de hersenen.

Hoofdstuk 6. De individuele verschillen in de farmacokinetiek van de bestudeerde ataractica komen sterk naar voren in de experimenten met honden. De isotoop-verdunningsanalyse maakt nauwkeurige meting van de plasmaconcentratie mogelijk. Biofarmaceutische aspecten zijn besproken. De verbindingen blijken in het algemeen snel omgezet te worden. De gevormde metabolieten bezitten een langere biologische halfwaardetijd dan de oorspronkelijke stof. De fictieve verdelingsvolumina van de geneesmiddelen uit de meprobamaat-groep welke berekend werden op basis van de plasmawaterconcentratie in een één-compartimentsysteem, blijken grote verschillen te vertonen in overeenstemming met de lipofiele eigenschappen. Berekend op basis van de plasmaconcentratie zijn deze verschillen gering.

De grote individuele variatie en het gecompliceerde verloop van de concentratie, die bij de vertegenwoordigers van de diazepine-groep geconstateerd werden, maakten het nog niet mogelijk nauwkeurige kinetische berekeningen uit te voeren. De veronderstelling, dat het fictieve verdelingsvolume in de praktijk een constante individuele waarde heeft, onafhankelijk van de gegeven dosis, moet betwijfeld worden gezien de potentiële grootte van dit volume. Nader onderzoek is echter noodzakelijk.

De eiwitbinding van chloordiazepoxide en van diazepam bij de farmacologisch werkzame plasmaconcentraties bedraagt resp. $\pm$ 90 % en $\pm$ 95 %. De beperkende invloed die de eiwitbinding op de verdeling van deze geneesmiddelen zou kunnen hebben wordt grotendeels gecompenseerd door de snelle en grote uitwisseling met de lipofiele weefsels van het lichaam. Gegevens verkregen bij de hond vormden de inleiding tot de analyse van diazepam bij de mens met behulp van gaschromatografie.

Hoofdstuk 7. Bij de mens worden in tegenstelling tot bij de muis en de hond veel langere biologische half-waardetijden van diazepam geconstateerd. Daar deze lange $t\frac{1}{2}$ aanleiding kan zijn tot cumulatie, zijn de plasmaconcentraties over lange periodes gevolgd. Na ongeveer 4 dagen wordt een cumulatieplateau bereikt. Grote individuele variaties en grillig verloop van de concentratie, waarschijnlijk als gevolg van enterale processen, zijn geconstateerd. N-desmethyldiazepam wordt als de belangrijkste metaboliet in plasma aangetroffen en heeft eveneens de neiging tot cumuleren bij chronisch gebruik. De concentratie aan metaboliet stijgt tot ongeveer gelijke hoogte als diazepam. Een gaschromatografische analyse methode in biologisch milieu van een aantal diazepines is beschreven.

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STELLINGEN

1. Bij de bepaling van diazepam in plasma wordt door DE SILVA en medewerkers ten onrechte verondersteld dat geen oxydatieproducten aanwezig zijn.

DE SILVA, J. A. F., SCHWARTZ, M. A., STEVANOVIC, V., KAPLAN, J. en D'ARCONTE, L. *Anal. Chem.*, 1964, *36*, 2099.

2. Het maagdarmkanaal draagt voor een belangrijk gedeelte bij aan het fictieve verdelingsvolume van psychofarmaca. Met de depotfunctie van het maagdarmkanaal voor deze geneesmiddelen dient rekening gehouden te worden bij de behandeling van overdoseringen en bij de potentiëring door o.a. alcohol.

3. De experimenten van KARE en medewerkers zijn niet bewijzend voor een directe transportweg tussen de mondholte en de hersenen.

a. — KARE, M. R., SCHECHTER, P. J., GROSSMAN, S. P. en ROTH, L. J. *Science*, 1969, *163*, 952.

b. — MALLER, O., KARE, M. R., WELT, M. en BEHRMAN, H. *Nature*, 1967, *213*, 713.

4. De concentratieverhouding in urine van oestrogenen- en 17-hydroxycorticosteroïden kan een waardevolle indicatie zijn voor de predispositie voor het ontstaan van varicosis.

ILCA, S., DODICA, C. en IOANAVICI, Z. Proc. 3rd Congress on Phlebology, Amsterdam, 1968. Noord Nederlandse Uitgeverij, Stenfert, Meppel, 1969.

5. Het is aan twijfel onderhevig of de moleculair gewichtsschattingen volgens SHAPIRO en medewerkers in SDS-bevattende polyacrylamide gels betrekking hebben op polypeptide ketens.

a. — SHAPIRO, A. L., VINUELA, E. en MAIZEL, J. V., Jr. *BBRC*, 1967, *28*, 815.

b. — SHAPIRO, A. L. *Invest. Ophtalm.*, 1968, *7*, 462.

6. Tabletten van acetylsalicylzuur met vertraagde afgifte hebben geen zin wanneer de verlengde werkzaamheid van dit geneesmiddel beoogd wordt.

a. — WISEMAN, E. H. en FEDERICI, N. J. *J. Pharm. Sci.*, 1968, *57*, 1535.

b. — ROWLAND, M. en RIEGELMAN, S. *J. Pharm. Sci.*, 1968, *57*, 1313.

7. De dosis afhankelijke eliminatiesnelheid van het anticoagulans bishydroxycoumarine laat zich niet verklaren met behulp van het farmacokinetische model met drie compartimenten zoals toegepast door NAGASHIMA en medewerkers.

a. — NAGASHIMA, R., LEVY, G. en NELSON, E. *J. Pharm. Sci.*, 1968, *57*, 58.

b. — NAGASHIMA, R., LEVY, G. en O'REILLY, R. A. idem, 1968, *57*, 1888.

8. In de magistrale receptuur wordt onvoldoende aandacht besteed aan de biofarmaceutische aspecten der voorschriften.

9. Het is niet geoorloofd de dosering van sterk lipofiele geneesmiddelen vast te stellen op basis van leeftijd, lichaamsgewicht of lichaamsoppervlak.

10. Om de toepassing van nieuwe materialen en technieken in de beeldende kunst mogelijk te maken dient ruimere materiële en financiële steun beschikbaar gesteld te worden.

E. VAN DER KLEIJN 1 juli 1969